(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 October 2002 (24.10.2002)

PCT

(10) International Publication Number
WO 02/083840 A2

(51) International Patent Classification[7]: C12N

(21) International Application Number: PCT/US02/10647

(22) International Filing Date: 3 April 2002 (03.04.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/281,275 3 April 2001 (03.04.2001) US

(71) Applicant *(for all designated States except US)*: **ARIZEKE PHARMACEUTICALS, INC.** [US/US]; 6828 Nancy Ridge Drive, Suite 400, San Diego, CA 92121 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SHERIDAN, Philip, L.** [US/US]; 10266 Wateridge Circle #228, San Diego, CA 92121 (US). **HOUSTON, Lou, L.** [US/US]; 327 Pine Needles Drive, Del Mar, CA 92104 (US).

(74) Agents: **WARBURG, Richard, J.** et al.; Foley & Lardner, P.O. Box 80278, San Diego, CA 92138-0278 (US).

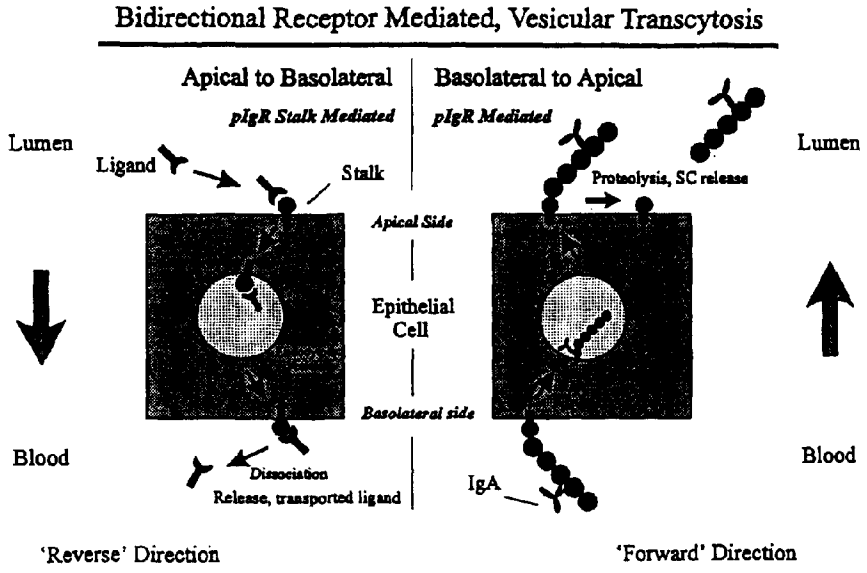
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *without international search report and to be republished upon receipt of that report*

*[Continued on next page]*

(54) Title: COMPOSITIONS AND METHODS FOR TRANSEPITHELIAL TRANSPORT OF MEMBRANE-BOUNDED VESICLES AND VIRIONS




(57) Abstract: The invention provides compositions and methods for the delivery of membrane-bounded vesicles such as liposomes and the virions of enveloped viruses, as well as nucleocapsid virions into, through, out of and around, epithelial cells and layers and, optionally, to an intracellular location. Such membrane-bounded vesicles and virions are used to deliver therapeutic agents in general. Certain aspects of the invention are particularly well suited for the delivery of therapeutic nucleic acids, i.e., gene therapy.

WO 02/083840 A2

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# COMPOSITIONS AND METHODS FOR TRANSEPITHELIAL TRANSPORT OF MEMBRANE-BOUNDED VESICLES AND VIRIONS

## FIELD OF THE INVENTION

The invention is drawn to compositions and methods for the delivery of membrane-bounded vesicles and virions into, through, out of and around, epithelial cells and layers and, optionally, to an intracellular location. Such vesicles and virions are used to deliver therapeutic agents in general. Certain aspects of the invention are particularly well suited for the delivery of therapeutic nucleic acids, i.e., gene therapy.

## BACKGROUND OF THE INVENTION

The following description of the background of the invention is provided simply as an aid in understanding the invention and is not admitted to describe or constitute prior art to the invention.

Therapeutic drugs can be introduced into the body using a variety of formulations and by various of routes of administration. For many reasons, a preferred route of administration is one that is non-invasive, i.e, does not involve any physical damage to the body. Generally, physical damage of this type results from the use of a medical device, such as a needle, to penetrate or breach a dermal surface or other external surface of an animal. Invasive routes of administration include, for example, surgical implants and injections. Injections can be intravascular, intrathecal or subcutaneous, all of which have undesirable features. Non-invasive routes of administration include uptake from the gastrointestinal tract as well as non-invasive parenteral (i.e, other than gastrointestinal) routes such as, e.g., inhalation therapy.

Progress in the field of molecular biology has been so rapid that it has created opportunities to develop therapeutic agents that were unimaginable a generation ago. Examples of such therapeutic agents are purified proteins and polypeptides, particularly those produced via in vitro synthesis or recombinant DNA technology, and gene therapy agents (GTAs). Presently, there are few, if any, formulations for the administration of proteins and GTAs, particularly in the case of non-invasive routes of administration and formulations therefor. The enormous therapeutic

potential of purified proteins and gene therapy agents, for a variety of reasons, has not been fully realized. One limitation is the paucity of compositions and methods for the non-invasive administration of purified proteins and GTAs.

Preferably, formulations and methods for the delivery of purified proteins and GTAs would preferentially or exclusively deliver these therapeutic agents to predetermined target cells and not to other cell types. A "target cell" is a specific type of cell found within or derived from an organism that it is desired to deliver a purified protein, GTA, or other biologically active molecules.

Current delivery systems for purified proteins and GTAs are not optimal in these aspects. Preferably, purified proteins and GTAs are delivered from the site of administration into the appropriate tissue and target cell. For gene therapy and, in some instances purified proteins, the therapeutic agent must traverse intracellular compartments in order to reach its site of action, i.e., the nucleus of the cell. Preferably, gene delivery vehicles satisfy two major criteria: the genetic delivery vehicle should circumvent extracellular barriers to reach target cells (extracellular targeting), and then traverse several target cell membranes and intracellular compartments to establish nuclear localization where transgene expression can occur (intracellular targeting).

The use of receptor-mediated cell targeting for purified proteins and GTAs has been suggested. For in vivo administration, directing purified proteins and GTAs to specific tissues or cells would have the advantages of (1) decreasing exposure to GI tract luminal proteases (following oral delivery), (2) decreasing exposure to complement defense components in the blood (during intravenous delivery), (3) minimizing dilution effects and inactivation of purified proteins and GTAs due to binding to non-productive cell types, and (4) minimizing the amount of purified proteins or expression of GTAs at sites distant from the site of interest, thereby minimizing potentially harmful toxic effects.

One strategy may also take advantage of the ligand binding and internalization enhancement as two separate processes, utilizing one aspect of the ligand for targeting and a separate property for internalization. In this regard, the targeting of cell biology of specific cell types, or specific cell surface receptors, also plays an important role for efficient targeting and gene delivery. Thus, the cellular targeting

ligand coupled to the nonviral (transgene DNA) or viral vector may be used to target only the corresponding cell, and the subsequent internalization can be achieved by the cell's normal mechanisms of membrane internalization (turnover) or endocytosis. For example, enveloped viruses (such as retroviruses) fuse with the cell membrane triggering the release of the viral core into the cell cytoplasm (Figure 6), whereas non-enveloped viruses (such as adenovirus) bind to receptors and are internalized via coated pits (Figure 7).

Compounds are trafficked into, out from and within a cell by various molecules. "Endocytosis" is a general term for the process of cellular internalization of molecules, i.e, processes in which cells takes in molecules from their environment, either passively or actively. "Exocytosis" is a general term for processes in which molecules are passively or actively moved from the interior of a cell into the medium surrounding the cell. "Transcytosis" is a general term for processes in which molecules are transported from one surface of a cell to another.

In general, endocytosis occurs by at least two known mechanisms: clathrin-dependent receptor-mediated and clathrin-independent endocytosis (Figure 8). Clathrin-dependent receptor-mediated endocytosis involves ligand binding to the cell surface receptor, clustering of the ligand-receptor complexes in clathrin-coated pits, invagination into the cell, budding off of the coated pits to form intracellular coated vesicles, and maturation into endosomes. Within the endosomes ligands and receptors are sent to their appropriate cellular destinations. Clathrin-independent mechanisms involving uncoated pits include phagocytosis, pinocytosis, potocytosis and transcytosis. The endocytotic pathways described above can be used to enhance delivery of foreign genes into cells. Many receptors contain protein motifs in their cytoplasmic domains that act as recognition sequences for initiating enhanced intracellular uptake of macromolecules, and the interaction of the ligand with the receptor increases the rate of internalization.

Active endocytosis, exocytosis and transcytosis typically involve or are mediated by receptors, molecules that are at least partially displayed on the surface of cells. Receptors have varying degrees of specificity; some are specific for a single molecule (e.g., a receptor specific for epidermal growth factor; or a receptor that specifically recognizes $Ca^{++}$); some are semi-specific (e.g., a receptor that mediates

the cellular internalization of many members of a family of cellular growth factors, or a receptor that recognizes $Ca^{++}$, $Mg^{++}$ and $Zn^{++}$); or of limited specificity (e.g., a receptor that mediates the cellular internalization of any phosphorylated protein, or a receptor that recognizes any divalent cation). Other types of molecules that can cause or influence the entry of molecules into cells include, e.g., cellular pores, pumps, and coated pits. Pores such as gated channels and ionophores form a channel that extends through the cellular membrane and through which certain molecules can pass. Cellular pumps exchange one type of molecule within a cell for another type of molecule in the cell's environment. Coated pits are depressions in the cellular surface that are "coated" with bristlelike structures and which condense to surround external molecules; the condensed coated pits then "pinch off" to form membrane-bounded, coated vesicles within the cell.

Molecules that cause, influence or undergo endocytosis, exocytosis and/or transcytosis can do so constitutively, i.e, at all times, or regulated, i.e, for example, only under certain conditions or at specific times. Some such molecules can only mediate and/or undergo endocytosis, whereas some mediate and/or undergo transcytosis as well as endocytosis. Moreover, some such molecules are present in all or most cells (i.e, are ubiquitous), or are present mostly or only in certain tissues (i.e, are tissue-specific) or particular cell types.

The lack of compositions and methods causing, enhancing, mediating or regulating the endocytosis of therapeutic, diagnostic or analytical compounds and compositions hinders or prevents various uses of such compounds. In particular, the full therapeutic potential of many compounds could be realized if they were taken up by cells lining the gastrointestinal tract, as one could then formulate pills or tablets for the administration of therapeutic agents to patients. Typically, pills and other formulations for the oral delivery, and suppositories for the rectal delivery, of therapeutic agents to the gastrointestinal tract result in better patient compliance, and less use of medical resources, as opposed to other delivery modalities such as, e.g., intravenous administration. Similarly, the therapeutic potential of many compounds could be realized if they were taken up by cells lining the respiratory tract, including the nasal cavity; cells lining the gastrointestinal tract; vaginal surfaces; on dermal surfaces; and ocular surfaces and buccal surfaces (see Sayani et al., Crit. Rev. Ther.

Drug Carrier Systems 13:85-184, 1996) . Attempts to develop oral delivery formulations for proteins are discussed by Wang (J. Drug Targeting 4:195-232, 1996), Sinko et al. (Pharm. Res. 16:527, 1999) and Stoll et al. (J. Controlled Release 64:217-228, 2000).

In addition to the need for compositions and methods for the entry of biologically active molecules into cells, there is a further need for compositions and methods for causing, enhancing, mediating or regulating, or that control the direction of, transcytosis. Transcytosis is the general term given for processes whereby molecules, including biologically active molecules, move through a cell, generally from one side or surface of a cell to another.

Degradation and inefficient absorption of compounds delivered by conventional means further reduces the efficacy of those compounds. The ability to utilize alternative delivery pathways, target particular cells and tissues for delivery, improve the retention and absorption of therapeutic compounds to be delivered, and protect the therapeutic agent compound during delivery, are desirable pharmaceutical attributes.

Limitations vis-à-vis cellular transport of molecules are present both in vitro (e.g., in cellular cultures) and in vivo (e.g., in animals). Such limitations prevent or limit the therapeutic, diagnostic and/or analytical uses as of various compounds and compositions in an animal, including a mammal which may be a human. Such uses are described herein.

One example of a molecule that undergoes or mediates endocytosis, exocytosis as well as forward and reverse transcytosis is the polymeric immunoglobulin receptor (pIgR). The following information regarding pIgR is provided to assist in understanding the background of the invention.

Typically, pIgR molecules are displayed on epithelial cells. Epithelial cells line the interior of organs that have enclosed, semi-enclosed or compartmentalized spaces. The interior (e.g., canals, ducts, cavities, etc.) of such organs is genericaly referred to as the lumen. The lumen of a particular organ may have a specific name, e.g., the gastrointestinal lumen, pulmonary lumen, nasal lumen, nasopharyngeal lumen, pharyngeal lumen, buccal (within the mouth) lumen, sublingual (under the tongue) lumen, vaginal lumen, urogenital lumen, ocular lumen, or tympanic lumen.

See, for example, Fahey et al., Immunol. Invest. 27:167-180, 1998; Brandtzaeg, J. Reprod. Immunol. 36:23-50, 1997; Kaushic et al., Biol. Reprod. 57:958-966, 1997; Richardson et al., J. Reprod. Immunol. 33:95-112; Kaushic et al., Endocrinology 136:2836-2844, 1995. Some of these might also be characterized as surfaces, e.g., the ocular surface.

Adjacent epithelial cells are connected by tight junctions. Disruption of tight junctions allows agents within the lumen, which often has an opening to the external environment of an animal, to penetrate into the body. Although such agents might include therapeutic agents, entry into the body via a disrupted tight junction is not specific; undesirable agents (e.g., bacteria, viruses, toxins and the like) will also be taken into the body. Due to this lack of specificity, as well as other factors, disruption of tight junctions for drug delivery purposes is generally not feasible and would, in any event, have many potential undesirable side effects.

Epithelial cells have two distinct surfaces: the apical side, which faces the lumen and is exposed to the aqueous or gaseous medium present therein; and an opposing basolateral (a.k.a. basal lateral) side that rests upon and is supported by an underlying basement membrane. The tight junctions between adjacent epithelial cells separate the apical and basolateral sides of an individual epithelial cell.

Epithelial cells are said to have polarity, that is, they are capable of generating gradients between the compartments they separate (for reviews, see Knust, Curr. Op. Genet. Develop. 10:471-475, 2000; Matter, Curr. Op. Genet. Develop. 10:R39-R42, 2000; Yeaman et al., Physiol. Rev. 79:73-98, 1999). This polarity reflects that fact that the cell has distinct plasma membrane domains (apical and basolateral) having distinct transport and permeability characteristics. For example, the apical side often contains microvilli for the adsorption of substances from the lumen, and, in ciliated cells, cilia are found on the apical membrane. As another example, the $Na^+/K^+$-ATPase pump is characteristically found only on the basolateral membrane.

Figure 1 shows the pathways of cellular transport involving the pIgR protein, which undergoes or mediates endocytosis, exocytosis as well as forward and reverse transcytosis, in epithelial cells. Molecules of pIgR are typically displayed on the surfaces of epithelial cells and direct the trafficking of immunoglobulin (IgA) molecules. Other classes and species of immunoglobulins may also be trafficked.

The right side of Figure 1 illustrates the "forward" (i.e, basolateral to apical) transcytosis of pIgR molecules, whereas "reverse" (apical to basolateral) transcytosis is shown on the left side of the figure.

Forward transcytosis is the best characterized biological function of pIgR, and serves to convey protective antibodies (IgA and IgM immunoglobulins) from the circulatory system to the lumen of an organ. In forward transcytosis, pIgR molecules displayed on the basolateral side of the cell bind IgA molecules in the bloodstream, and pIgR:IgA complexes are then endocytosed, i.e, taken up into the cell and into a vesicle. The pIgR:IgA complexes are transported to the apical side of the cell, where they are displayed on the cell surface. Delivery of IgA into the lumen occurs when the pIgR portion of a pIgR:IgA complex is cleaved, i.e, undergo proteolysis. This event separates the pIgR molecule into two components: the "secretory component" (SC), which is released into the lumen, and which remains bound to IgA in order to protect IgA from degradation, and the "stalk," which remains displayed, at least temporarily, on the apical surface of the cell.

Surprisingly, ligands bound to stalks displayed on the apical side of a cell can undergo reverse transcytosis, i.e, transcytosis in the opposite direction of forward transcytosis, i.e, from the apical side of a cell to its basolateral side. In reverse transcytosis, pIgR molecules or portions thereof move from the apical surfaces of cells that line the lumen of an organ to the basolateral surfaces of these cells. In theory, pIgR-mediated reverse transcytosis could be used to deliver agents from a lumen (e.g., the interior of the gut or the airways of the lung) to the circulatory system or some other interior system, organ, tissue, portion or fluid of the body including by way of non-limiting example the lymphatic system, the vitreous humor, etc. For example, as is shown in Figure 1, a compound having a element that binds to a portion of pIgR that undergoes reverse transcytosis could, due to its association with the pIgR stalk, be carried to the basolateral side of a cell, where it would be contacted with and/or released into the bloodstream.

Evidence has been presented that forward transcytosis is mediated by a vesicular process (Apodaca et al., J. Cell Biol. 125:67-86, 1994; Mostov, Annu. Rev. Immunol. 12:63-84, 1994). Although not wishing to be bound by any particular theory, Figure 1 shows a similar vesicular mediated transport mechanism for reverse

transcytosis. Figure 1 is not intended to imply that such a mechanism actually exists because evidence to this fact is not available; the vesicular nature of reverse transcytosis is only a hypothesis based on what is known about forward transcytosis.

The polyimmunoglobulin receptor (pIgR) is reviewed by Mostov and Kaetzel, Chapter 12 in: Mucosal Immunology, Academic Press, 1999, pages 181-211 (1999).

U.S. Patent No. 6,020,161 to Wu et al. is drawn to pIgR polypeptides and polynucleotides that encode pIgR polypeptides.

U.S. Patent No. 5,484,707 to Goldblum et al. is drawn to methods for monitoring organ rejection in an animal based on the concentration of the free secretory component of (SC) pIgR.

Published PCT patent applications WO 98/30592 and WO 99/20310, both to Hein et al., and U.S. Patent 6,045,774 to Hiatt et al., are drawn to synthetic proteins that mimic IgA molecules and are thus associated with the proteolytically generated secretory component (SC) of pIgR.

U.S. Patent No. 6,072,041 to Davis et al. is drawn to fusion proteins that are directed to the secretory component of pIgR. The compositions of Davis et al. are stated to be transported specifically from the basolateral surface of epithelial cells to the apical surface.

Ferkol et al., Am. J.Respir. Crit. Care Med. 161:944-951, 2000, is stated to describe a fusion protein consisting of a sFv directed to the secretory component (SC) of human pIgR and an human alpha-(1)-antitrypsin.

U.S. Patent No. 6,042,833 to Mostov et al. is drawn to a method by which a ligand that binds to a portion of a pIgR molecule is thereby internalized into, or transported across, a cell expressing or displaying pIgR. Mostov et al. describes gene therapy modalities in which an adenoviral vector is conjugated to a poly-Lys stretch of amino acids that is fused to an antibody that directs reverse transcytosis. See also Guy et al., "High-Efficiency Gene Transfer Employing Adenovirus-Polylysine-DNA Complexes", Nat. Immun., Vol. 3, pp. 141-64, 1994. U.S. Patent 6,083,741, to Hart et al., entitled "Internalisation of DNA, Using Conjugates of Poly-lysine and an Intergrin Receptor Ligand," combines this technique with the use of an integrin receptor ligand.

U.S. patent application Serial No. 60/199,423 (attorney docket nos. 030854.0008, 057220-0201 entitled "Compositions Comprising Carriers and

Transportable Complexes" by Houston, L.L.), filed April 23, 2000, describes various pharmaceutical compositions that may be applied to compositions and methods of the present invention.

U.S. patent application Serial No. 60/192,197 (attorney docket no. 18062E-000900 entitled "Ligands Directed to the Non-Secretory Component, Non-Stalk Region of pIgR and Methods of Use Thereof" by Mostov, Keith E., and Chapin, Steven J.), filed March 27, 2000, describes the B region of pIgR and ligands directed to the B region of pIgR.

U.S. patent application Serial No. 60/192,198 (attorney docket no. 18062E-003000US entitled "Anti-pIgR Antibodies With Improved Transcytosis by Mostov, Keith E., Chapin, Steven J., and Richman-Eisenstat, Janice), filed March 27, 2000, is drawn to single chain Fv antibody fragments (sFv or scFv) directed to a specific epitope in the B region of pIgR. The application describes two single chain antibodies directed to pIgR named "sFv-5A" and "sFv-5AF," the latter of which is used in the studies described in the Examples.

Zhang et al. (Cell 102:827-837, 2000) states that pIgR translocates bacteria (specifically, Streptococcus pneumoniae) across nasopharyngeal epithelial cells. The bacterial translocation is reported to occur in the apical to basolateral (reverse) direction.

U.S. patent application Serial No. 60/237,929 (attorney docket nos. 030854.0009, 057220-0301) entitled "Genetic Fusions of pIgR Ligands and Biologically Active Polypeptides for the Delivery of Therapeutic and Diagnostic Proteins" by Houston, L.L., Glynn, Jacqueline M., and Sheridan, Philip L.), filed October 2, 2000, is drawn to fusion proteins comprising pIgR ligands and biologically active polypeptides.

U.S. patent application Serial Nos. 60/248,478 and 60/248,819 (attorney docket nos. 030854.0009.PRV2, 057220-0601 and 030854.0009.PRV3, 057220-0602) entitled "Protein Conjugates of pIgR Ligands for the Delivery of Therapeutic and Diagnostic Proteins" by Houston, L.L., and Hawley, Stephen), filed November 13, 2000 and November 14, 2000 respectively, are drawn to protein conjugates comprising pIgR ligands and biologically active polypeptides.

U.S. patent application Serial No. 60/266,182 (attorney docket No. 057220.0701 entitled "Compositions and Methods for Identifying, Characterizing, Optimizing and Using Ligands to Transcytotic Molecules" by Houston, L.L., and Sheridan, Philip L.), filed February 2, 2001, is drawn to the identification and use of transcytotic and transepithelial molecules.

U.S. patent application Serial No. 60/267,601 (attorney docket No. 057220.0401 entitled "Polyspecific Binding Molecules Having a Polymeric Immunoglobulin Receptor Binding Region" by Houston, L.L., and Sheridan, Philip L.), filed February 9, 2001, is drawn to multivalent, including but not limited to bispecific, ligands that specifically bind a portion of pIgR and a biologically active compound.

U.S. patents Nos. 5,844,107; 5,877,302; 6,008,336; and 6,077,835, all to Hanson et al. and entitled "Compacted Nucleic Acids and Their Delivery to Cells," are drawn to the introduction of DNA into cells via complexes of polycations.

## SUMMARY OF THE INVENTION

In one aspect, the invention provides a fusion protein comprising a transepithelial delivery element from a first protein and a transmembrane domain from a second protein.

A "transmembrane domain" is an amino acid sequence that provides for the insertion of a protein into a bilayer lipid membrane or anchoring thereto. The process of insertion can occur in vivo (e.g., during translation and export via the Golgi apparatus) or in vitro. At least two types of in vitro insertion processes are known: reconstitution, by which a purified protein is introduced into membranes via biochemical and/or biophysical manipulations; and spontaneous insertion by proteins that insert into membranes upon contacting them, apparently without the help of other factors.

A "transepithelial delivery element" is one that undergoes any type of active or passive transport across an epithelial cell or through an epithelial barrier. Such elements confer the properties of being able to penetrate the epithelial cell layer, to undergo paracellular transport (e.g., through tight gap junctions), and/or to transcytose an epithelial cell. Preferred transepithelial delivery element are capable

of being displayed on the exterior of a membrane-bounded vesicle. A membrane-bounded vesicle includes, but is not limited to, a liposome and an enveloped virion. Another type of virion is referred to as "non-enveloped" as its genome is contained within a protein coat rather than a lipid membrane.

The transepithelial delivery element and the transmembrane domain are preferably both derived from proteins that are endogenous to a single species, but may be derived from proteins that are endogenous to two different species.

In a related aspect, the fusion protein further comprises a polypeptide selected from the group consisting of a detectable polypeptide and a cell-specific targeting element.

A "targeting element" or "ligand" is any molecule, compound or moiety that is directed to (specifically binds) a molecule to which it is targeted. As used herein, the terms "ligand" and "targeting element" are synonymous. A ligand may be any type of molecule that is capable of binding to a preselected target molecule. In the present disclosure, amino acid sequences (polypeptides, oligopeptides) are preferred targeting elements.

A targeting element is said to be "cell-specific" if it is directed to a molecule that is exclusively or preferentially displayed on the surface of a cell of a particular cell type or tissue. Cell-spefic displayed molecules include, but are not limited to, receptors and surface antigens.

By "detectable" it is meant that a composition or moiety is detectable by spectroscopic, photochemical, biochemical, immunochemical, electromagnetic, radiochemical, or chemical means such as fluorescence, chemifluoresence, or chemiluminescence, or any other appropriate methodologies. In the present disclosure, preferred detectable moieties are detectable amino acid sequences (polypeptides, oligopeptides). These include, by way of non-limiting example, a green fluorescent protein (GFP) or a derivative thereof, which can be detected by their ability to fluoresce; or an epitope for which antibodies are available (including but nor limited to commercially avalable ones such as c-myc epitope and the FLAG-tag), which may thus be detected using any of a variety of immunoassays such as enzyme-linked immunosorbent assay (ELISA) or a radioimmunoassay (RIA).

In a related aspect, the transepithelial delivery element is a targeting element directed to a protein selected from the group consisting of a pIgR molecule, a pIgR stalk molcule, and a pIgR secretory component molecule. Such elements confer the property of being able to undergo apical or basolateral endocytosis, apical or basolateral exocytosis, and/or forward or reverse transcytosis in cells displaying a pIgR molecule, a pIgR stalk molcule, and/or a pIgR secretory component molecule. By way of non-limiting example, the pIgR may be a simian pIgR, a murine pIgR, a rodent pIgR, a rabbit pIgR, a bovine pIgR, or a human pIgR.

A molecule is said to be "displayed" by a cell when it is present on the surface of a cell in such a way so as to be accesible to outside factors. Different cell types may display different proteins, and this feature can be used to identify or target a specific set of cells within a population of cells.

In one aspect, the invention provides a membrane-bounded vesicle comprising a fusion protein that comprises a transepithelial delivery element from a first protein and a transmembrane domain from a second protein, wherein the epithelial delivery element is displayed on the exterior of said membrane-bounded vesicle.

A "membrane-bounded vesicle" is any semi-permeable enclosed volume that has as its boundary a membrane, e.g., a lipid bilayer. Non-limiting examples of membrane-bounded vesicles include virions and liposomes.

A membrane-bounded vesicle of the invention may contain ("be loaded with") a biologically active molecule, which may be a biologically active nucleic acid, protein or small molecule. Preferably, such vesicles are themselves biologically active and/or serve as delivery agents for biologically active molecules.

The term "biologically active" (synonymous with "bioactive") as it is used herein indicates that a molecule or moiety itself has a biological effect, or that it modifies, causes, promotes, enhances, blocks, reduces, limits the production or activity of, or reacts with or binds to an endogenous molecule that has a biological effect. Preferred biological active compositions have a therapeutic effect.

A "biological effect" may be but is not limited to one that stimulates or causes an immunreactive response; one that impacts a biological process in an animal; one that impacts a biological process in a pathogen or parasite; one that generates or causes to be generated a detectable signal; and the like. Biologically active

conjugates may be used in therapeutic, prophylactic and diagnostic methods and compositions. Biologically active compounds act to cause or stimulate a desired effect upon an animal. Non-limiting examples of desired effects include, for example, preventing, treating or curing a disease or condition in an animal suffering therefrom; limiting the growth of or killing a pathogen in an animal infected thereby; augmenting the phenotype or genotype of an animal; stimulating a prophylactic immunoreactive response in an animal; or diagnosing a disease or disorder in an animal.

Biologically active nucleic acids include, but are not limited to, catalytic nucleic acids, *e.g.*, ribozymes; structural nucleic acids, *e.g.*, ribosomal RNA (rRNA); transfer RNA (tRNA); antisense nucleic acids, *e.g.*, antisense oligonucleotides; aptamers; nucleic acids decoys, *e.g.*, dsDNA comprising sequences to which DNA-binding proteins specifically bind; and expression elements that direct the *in vivo* production of a biologically active nucleic acid or polypeptide. The biologically active nucleic acids of the invention may be of any shape, form or topology including, but not limited to, double-stranded (ds), including A-, B- and Z-DNA; single-stranded (ss); mixed ds and ss; linear; circular; hybrid (*e.g.*, RNA:DNA hybrids); supercoiled; compacted; nicked; complexed with other nucleic acids and/or polypeptides; etc.

The term "expression element" refers to a nucleic acid sequence comprising at least one nucleic acid sequence operably linked to the appropriate expression elements. By the term "operably linked" it is meant that the gene products encoded by the non-vector nucleic acid sequences are produced from the expression element *in vivo*. The term "gene product encoded" refers to either a nucleic acid (the product of transcription, reverse transcription, or replication) or a polypeptide (the product of translation) that is produced using the non-vector nucleic acid sequences as a template.

Biologically active polypeptides include, but are not limited to, (1) endogenous polypeptides that are missing, deficient, mutated or underexpressed in a patient suffering from a particular disease or disorder; (2) endogenous polypeptides that can be overexpressed to achieve a biological effect; and (3) exogenous proteins. Some therapies involving polypeptides of type (1) are designed so as to be therapeutic for

inborn errors of metabolism and include, by way of non-limiting example, enzyme replacement (*e.g.*, Factor IX in the case of hemophilia B, and phenylalanine hydroxylase in the case of phenylketonuria) therapy, and protein or factor replacement (*e.g.*, Factor VIII in the case of hemophilia A, and insulin in the case of Type I diabetes) therapy. See, *e.g.*, Dai *et al., Proc. Natl. Acad. Sci. USA 89*:10892-10895, 1992; and Wang *et al., Proc. Natl. Acad. Sci. USA 96*:3906-3910, 1999.

An RNA molecule may be an mRNA molecule that encodes a biologically active polypeptide, or a template for reverse transcription. A DNA molecule may be one that, when transcribed, directs the production of an mRNA molecule that encodes a biologically active polypeptide, a ribozyme, or an antisense transcript.

A biologically active polypeptide is, by way of non-limiting example, a polypeptide that is of deficient quantity or biological activity in the cells of an animal in need thereof, a negative dominant mutant of a polypeptide in the cells of an animal in need of a reduced quantity or activity of said polypeptide (see, e.g., Bevec et al., Proc. Natl. Acad. Sci. USA 89:9870-9874, 1992); or a polypeptide that is a regulator of gene expression. Non-limiting examples of biologically active small molecules include organic compounds, peptidomimetics, oligopeptides, oligonucleotides, aptamers and mixtures and conjugates thereof.

In one aspect, the invention provides a membrane-bounded vesicle comprising a fusion protein that comprises a transepithelial delivery element from a first protein and a transmembrane domain from a second protein, wherein the transmembrane domain is that of an envelope protein of a virus.

Viral envelope proteins are described in the detailed description of the disclosure, but may generally be described as proteins, which may be transmembrane proteins, that are displayed on the surface of an enveloped virion. The envelope protein may be an envelope protein that is derived from an ecotropic virus, a xenotropic virus, or an amphotropic virus. Ecotropic viruses replicate in the cells of the host species and sometimes in those of closely related species. Xenotropic viruses infect cells of many other species with varying efficiency and, upon introduction into the cells of an animal of interest are expressed but are unable to infect cells of that animal. Amphotropic viruses are able to infect the cells of their host and the cells of

other species. For example, murine amphotropic viruses were originally derived from the exogenous viruses of wild mice and form the basis of the most widely used packaging cell lines that release virions capable of infecting human cells.

In a related aspect, the envelope protein is one found in a pseudotyped virus. "Pseudotyping" refers to the incorporation of specific heterologous envelopes from different viruses to produce high titer viral vectors with broad host-ranges. Pseudotyping has been used to target viral vectors for entry into cell types of alternative species, but not, however, necessarily in a tissue or cell type specific manner. Attempts have been made to expand the host range of a viral gene delivery vehicle into specific human cells by targeting cell- or tissue-specific receptors (reviewed by Ameri and Wagner, chapter 8, Gene Therapy: Therapeutic Mechanisms and Strategies) through receptor-specific ligands. These efforts have been undertaken by incorporating either the natural ligand of these receptors, or synthetically derivatives thereof, that are internalized via receptor-mediated internalization mechanisms. Natural ligands may represent various macromolecules such as proteins (transferrin), lipids (LDL), peptides such as growth factors (EGF, IGF-1, FGF-2) and hormones (growth hormone), or sugar derivatives. Examples of synthetic ligand derivatives can represent small molecules such as folic acid or larger macromolecules such as antibodies or antibody derivatives (sFv's). Some ligands are very specific in targeting certain tissues or cells, whereas others are not. Additionally, some ligands are efficiently internalized while others may be internalized slowly or not at all. In cases where targeting is not very tissue or cell type specific, strategies incorporating cellular or tissue-specific enhancer or promoter DNA regulatory elements into the viral expression vector have been used to "transcriptional" targeting or restrict cell type specific expression of the therapeutic transgene.

By way of non-limiting example, pseudotyped retrovirions may be produced using packaging cell lines that produce gag and pol proteins from one virus and env proteins from a second virus. For example, nontargeted, pseudotyped retrovirions based upon MuLV (murine leukemia virus) and carrying the envelope protein of the highly promiscuous vesicular stomatis virus (VSV) have been described (Yee, J. K. et. al., Proc. Natl. Acad. Sci. USA, 91:9564-9568 (1994)). These MuLV/VSV pseudotyped retrovirions have a wide infection spectrum and are able to infect even

fish cells. Presumably, when such vectors are used for gene therapy, they are capable of infecting many non-target cells.

Pseudotyped retroviral vectors based upon MoMuLV (MLV) and carrying the envelope of gibbon ape Leukemia virus (GaLV SEATO-MoMuLV hybrid virion) or the HTLV-I envelope protein (HTLV-I MoMuLV hybrid virion) have been described (Wilson, C. et. al., J. of Virology, 63(5):2374-2378 (1989)). GaLV SEATO-MoMuLV hybrid particles were generated at titers approximately equivalent to those obtained with the MoMuLV particles, and the infection spectrum correlates exactly with the previously reported in vitro host range of wild type GaLV SEATO, i.e., bat, mink, bovine and human cells.

In a related aspect, the fusion protein comprises the amino-terminal domain of an envelope protein of a retrovirus such as MuLV, including but not limited to a lentivirus such as feline or human immunodeficiency viruses (FIV and HIV, respectively). In a related aspect, the fusion protein comprises the surface protein domain (SU) of an envelope protein of a retrovirus.

In a separate aspect, the invention provides a fusion protein comprising a transepithelial delivery element and a polypeptide having an amino acid sequence found in a structural protein of a virus.

A viral structural protein may be, by way of non-limiting example, a capsid protein such as those that coat the genomes of viruses such as adenovirus, adeno-associated virus (AAV), Herpes Simplex Virus (HSV), or an alphavirus. Preferably, the transepithelial delivery element is displayed on the exterior of a non-enveloped virion.

As used herein, the term "cell" has its ordinary meaning in biology and further comprises cell-derived achromosomal membrane-bound entities, the latter including by way of non-limiting example RBC (red blood cells) and platelets. However, in no instances does the term "cell" encompass virions comprising membranes derived from cellular membranes. A cell may be a member of a culturable cell line derived from an animal; a cell that is part of an animal; a gamete of an animal; a zygote of an animal; a cell of an embryo; a cell of a fetus; and a cell that has been removed from an animal, embryo, or fetus.

The virions of the invention may be used to introduce biologically nucleic acids into the cells of gametes, zygotes, fetuses, embryos and other prenatal form of an animal. As used herein, an animal may be a human. However, although the virions of the invention may thus be used to introduce biologically active nucleic acids into whole humans (including adult and non-adult humans), human gametes and prenatal human forms (*e.g.*, zygotes, embryos, fetuses and the like), this statement should not be taken as indicating that the invention will be practiced in a manner that violates any legal restrictions or regulations regarding the transfer of nucleic acids into humans, human gametes, or prenatal forms of humans.

In a related aspect, the structural protein forms part of the nucleocapsid of an adenovirus. The entirety of the surface protein need not be present in the fusion protein; for example, the fusion protein might comprise the carboxy-terminus, the amino-terminus or the central domain of a capsid protein. Structural proteins include, as is described in detailed elsewhere herein, the fiber knob and/or penton base of an adenovirus; and a capsid protein such as the VP1, VP2 and VP3 capsid proteins of adeno-associated virus (AAV).

In related aspects the invention provides any of the preceding fusion proteins further comprising: a spacer and/or a tag and/or a protein purification element and/or nuclear localization elements.

As used herein, the term "and/or" has the meaning of "and, additionally or alternatively" or "and, in addition or in the alternative."

A "tag" is a detectable polypeptide as described above. A "spacer" is an amino acid sequence that is introduced between two parts of a polypeptide for the purpose of, e.g., reducing intramolecular steric hinderance, extending the "reach" of a functional moiety in a polypeptide, creating a cleavable sequence between two parts of a polypeptide, and the like. Such spacers may be relatively rigid, e.g., a poly-proline spacer, or relatively flexible, e.g., a poly-glycine spacer or a poly-glutamic acid spacer.

As used herein, "protein purification elements" are amino acid sequences that can be incorporated into a fusion protein in order to facilitate the purification or isolation thereof (e.g., a 6xHis-tag, which binds Nickel, glutathione-S-transferase, which binds glutathione, etc.) Protein purification elements include without limitation

secretion sequences that direct recombinantly produced proteins out of the host cell and into the cellular media.

As used herein, "nuclear localization elements" include any element that is required or expedites transport of the therapeutic nucleic acid of a virion to the nucleus. Such elements include, by way of non-limiting example, endosomal release peptides, such as adenovirus capsid peptides; membrane disruptive peptides, such as those found in influenza virus; a nuclear localization signal; and a DNA-binding domain.

In one aspect, the invention provides packaging cell lines that are used to produce virions that incorporate the preceding fusion proteins, and methods of producing such cell lines.

As is detailed in the disclsoure, a "packaging cell line" is one that produces non-infective (empty) virions unless a viral genome, or a nucleic acid derived therefrom, is introduced into the cell. Such cell lines are used to produce virions, i.e., non-infective viral particles that contain therapeutic nucleic acids. The virion proteins are supplied in trans by the packaging cell line and need not be the same as those found with the wildtype virus. Packaging a virally-derived, therapeutic nucleic acid into a non-wildtype virion results in a pseudotyped virion, as is explained in more detail elsewhere herein.

In one aspect, the invention provides methods for ex vivo treatments of cells using the fusion proteins, liposomes and virions of the invention.

By "ex vivo treatment" it is meant that cells are removed from a patient suffering from a disease and transfomed with a gene therapy vector. Successfully infected cells, which express a therapeutic nucleic acid or protein, are returned to the affected individual.

In one aspect of the invention, GTAs are used in pro-drug strategies.

By way of non-limiting example, the thymidine kinse (TK) gene of HSV-1 has a relaxed substrate specificity compared to endogenous human TK. A gene encoding the HSV-1 TK, or a mutant thereof, is delivered to cancer cells and, when they enter cells so transformed, nucleoside anlaogs (e.g., ganciclovir, GCV; acylovir, ACV) are phosphorylated, a step which leads to their development into nucleoside triphosphates. Thus, targeting of a HSV-TK-encoding GTA to, e.g., a cancer cell,

would increase the effective intracellular dose of GCV and/or ACV. Mutant HSV TK's with altered specificities may be particularly useful in some instances, and are described by Black et al., Proc. Natl. Acad. Sci. USA 93:3525-3529, 1996.

In related aspects, the invention provides pharmaceutical compositions comprising the membrane-bounded vesicles or virions of the invention. As is explained in more detail below, such pharmaceutical compositions may be used in various therapeutic and diagnostic methods.

The summary of the invention described above is not limiting and other features and advantages of the invention will be apparent from the following detailed description of the preferred embodiments, as well as from the claims.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows forward and reverse transcytotic pathways of the polyimmunoglobulin receptor (pIgR) in epithelial cells.

Figure 2 shows alignments of the amino acid sequences of pIgR homologs. Figure 2A, alignment of human, bovine, rat, mouse and rabbit pIgR molecules; Figure 2B, alignment of human, rabbit and simian pIgR molecules.; Figure 2C, alignment of amino acid sequences from the pIgR stalk regions of human, rabbit and simian pIgR molecules; 2D, alignment of human pIgR amino acid sequences with 2 clones of a simian pIgR molecule.

Figure 3 shows the amino acid sequence (SEQ ID NO:2) of the secreted form of the ScFv 5AF encoded by pSyn5AF. Symbols: Pelb leader, a leader sequence that directs secretion from *E. coli*; FLAG, FLAG epitope; linker, amino acid sequence $(GGGS)_3$; myc, c-myc epitope; 6 HIS, 6xHis tag; CDR, complementarity-determining region; FR, framework element; and the heavy and light chains of the scFv are indicated. The sequence of ScFv 5A is identical to that of Sc 5A with the exception that the 5th residue in the ScFv sequence is glutamine (Q) in 5A and leucine (L) in 5AF. The amino-terminal Pelb leader sequence is MKYLLPTAAAGLLLLAAQPAMA, and the carboxy terminal sequence is AAAEQKLISEEDLNGAAHHHHHH.

Figure 4 shows the partial amino acid sequence of a bacterial adhesion protein, CbpA (SEQ ID NO:__). Emboldened and underlined amino acid sequences indicate amino acid sequences that bind, or contain an element that binds, pIgR.

Figure 5 shows general characteristics of gene delivery systems.

Figure 6 is a schematic illustration of the retroviral life cycle. Retroviral infection is initiated by binding of the envelope glycoprotein to the RAM-1 cell surface receptor which triggers fusion between the viral and host membranes. The viral core is released into the cytoplasm, the viral RNA is transcribed by reverse transcriptase into dsDNA and the DNA integrated into the host genome. LTR sequences located at the termini of the integrated provirus direct the transcription of viral RNA encoding for the viral structural proteins Gag, Pol, Env as well as the viral genome. Viral core particles are assembled at the plasma membrane and package the viral RNA genome, and the viral particles bud from the plasmamembrane.

Figure 7 is a schematic illustration of adenoviral internalization and trafficking to the nucleus. Binding of the CAR receptor by the virus occurs through the knob domain of the fiber, which allows for interaction of the penton base with avb3/5 integrins and viral-receptor internalization via coated pits. Prior to fusion of the early endosome into sorting endosomes, the viral capsid undergoes a conformational change allowing escape of the virus into the cytoplasm. Microtubules carry the virus towards the nucleus, where only the DNA and terminal protein are inserted and assembled onto the nuclear matrix. Here the episomal establishment of the viral DNA allows for viral transcription.

Figure 8 shows is a schematic illustration of receptor mediated endocytosis. Receptor internalization via receptor-mediated endocytosis is a natural mechanism for transfer of materials into cells. It involves the binding of the ligand to a specific cell surface receptor, resulting in the clustering of ligand-receptor complexes in coated pits, which bud off from the membrane to form intracellular coated vesicles. These vesicles mature into endosomes, and within the endosomes, ligands and receptors are sent to there appropriate cellular destinations (lysosome, golgi apparatus, nucleus, apical or basal membranes).

Figure 9 shows packaging and vector producing cell line components. The packaging constructs provide all of the viral proteins in trans to the vector genome.

The retroviral vector plasmid codes for no viral proteins but contains all the necessary cis elements, and has been engineered to accommodate a selectable marker gene and the therapeutic gene of interest. Deletion of the packaging signal (ψ) from the packaging constructs prevents their incorporation into the viral particles.

Figure 10 shows a general strategy for the production of retroviral packaging and vector producing cell line components. Parental cell lines are characterized and selected based on their inherent ability to produce high viral titers. Parental cell lines are sequentially and stably transfected with the gag/pol and env packaging constructs, which provide all the viral structural proteins in trans, to generate stable packaging cell lines (PCLs). These stable packaging cells lines are then used to introduce the specifically engineered retroviral vector which will supply the viral genome to the vector particles. These vector producing cell lines (VPCLs) are stably selected and allow for the establishment of a stable cell bank for the specific VPCL carrying the desire therapeutic gene of interest.

Figure 11 shows retroviral vectors and various engineering derivatives. Regions in which genetic elements can be incorporated into the retroviral vector to enhance viral RNA genome expression, stability and safety are depicted in the illustration. SIN, self-inactivating vector.

Figure 12 shows various retroviral envelope genetic fusion strategies. (A), Schematic showing a retroviral envelope protein and how it is processed to generate the mature Env glycoprotein. Leader, leader peptide; SU, surface peptide; TM, transmembrane peptide. (B), Schematic illustrating different genetic chimera strategies to fuse pIgR-specific ligands to the SU or TM peptides of the retroviral Env protein for re-direction of the recombinant viral vector.

Figure 13 shows re-targeting strategies using chimeric retroviral envelope fusion molecules or bi-specific targeting ligands and is a schematic illustration of different genetic chimera strategies to create fusion proteins comprising pIgR ligands and the SU or TM peptides of the retroviral Env protein for re-direction of the recombinant retroviral vector. (A) wildtype Env, (B) SU N-terminal fusion, (C) TM N-terminal fusion, (D) an SU fusion protein where a small or large portion of the SU peptide replaced, and the optional incorporation of linkers and cleavable linkers, (E) a

schematic illustration showing a pIgR ligand fused to a bi-specific antibody or diabody which specifically recognizes an epitope on either the SU or TM peptides.

Figure 14 shows adenoviral genetic fusion strategies. A diagram of an adenoviral particle (top), and schematic illustrations of different genetic chimera strategies to fuse pIgR-specific ligands with a penton base, fiber, or knob protein domains for re-direction of the recombinant adenoviral vector. (A) a penton base fusion molecule, (B) a truncated fiber fusion molecule, (C) a full-length terminal fiber fusion protein (D) fusion of the pIgR ligand to the globular knob domain to create a fiber/knob-pIgR ligand fusion protein. Various linkers and cleavable linkers can optional be incorporated into these fusion proteins.

Figure 15 illustrates targeting of alphavirus-based gene therapy vectors; specifically, a strategy to re-target a recombinant Sindbis virus through the use of an E2 glycoprotein fusion protein.

Figure 16 is a schematic illustration depicting pIgR-specific bi-specific antibodies or diabodies with specificity for selected viral protein epitopes for re-targeting of viral vectors. A genetically engineered fusion protein comprising an antibody (or antibody derivative) that is directed to a viral protein epitope is fused to a pIgR-specific ligand

Figure 17 illustrates naked DNA vectors and targeted delivery strategies. Panel (A) is a schematic representation of genetically engineered pIgR binding proteins aimed at naked (plasmid) DNA delivery, in which the pIgR ligand would be fused in-frame to various protein domains for the facilitation of specific cellular functions such as DNA-binding (DNA-binding), endosomal escape (EEP, endosomal escape peptide), and nuclear transfer or residence (NLS, nuclear localization signal). These protein domains can be located in various orientations relative to the pIgR ligand, and different examples for each are shown. Panel (B) is a schematic illustration of a plasmid that is delivered by the fusion protein shown in (A). The plasmid contains a specific transcription factor DNA-binding site, general or tissue-specific enhancer and promoter cis transcription elements, an efficient polyadenylation signal (pA), an RNA cytoplasmic transport element (CTE), and/or additional RNA stability elements.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions and methods for the delivery of membrane-bounded vesicles and virions into, through, out of and around, epithelial cells and layers and, optionally, to an intracellular location. Such vesicles and virions are used to deliver therapeutic agents in general. Cetain aspects of the invention are particularly well suited for the delivery of therapeutic nucleic acids, i.e., gene therapy.

### I. STRUCTURE AND FUNCTION OF PIGR

A pIgR molecule has several structurally and functionally distinct regions that are defined as follows. It has been mentioned above that, in the art, a pIgR molecule is generally described as consisting of two different, loosely defined regions called the "stalk" and the "secretory component" (SC). A pIgR molecule binds polymeric immunoglobulins (IgA or IgM) on the basolateral side, and then transports the immunoglobulin to the apical side. Proteolyic cleavage of pIgR takes place on the apical side of an epithelial cell between the SC and the stalk, the former of which remains bound to and protects the immunoglobulins, and the latter of which remains bound to the apical membrane (see "Mucosal Immunoglobulins" by Mestecky et al. in: Mucosoal Immunology, edited by P.L. Ogra, M.E. Lamm, J. Bienenstock, and J.R. McGhee, Academic Press, 1999).

Particularly preferred pIgR molecule are those described in U.S. Patent 6,042,833, and the simian pIgR described in U.S. patent application Serial No. 60,266,182, attorney docket No. 057220.0701 entitled "Compositions and Methods for Identifying, Characterizing, Optimizing and Using Ligands to Transcytotic Molecules" by Houston, L.L., and Sheridan, Philip L., which was filed on February 2, 2001. However, it is understood that, in the context of this invention, pIgR also refers to any of that receptor's family or superfamily members, any homolog of those receptors identified in other organisms, any isoforms of these receptors, as well as any fragments, derivatives, mutations, or other modifications expressed on or by cells such as those located in the respiratory tract, the gastrointestinal tract, the urinary and reproductive tracts, the nasal cavity, buccal cavity, ocular surfaces, dermal surfaces and any other mucosal epithelial cells.

Preferred pIgR and pIgR-like proteins are those that direct the endocytosis or transcytosis of proteins into or across epithelial cells.

As used herein, the terms "secretory component" and "SC" refers to the smallest (shortest amino acid sequence) portion of an apical proteolyzed pIgR molecule that retains the ability to bind immunoglobulins (IgA and IgM). After proteolytic cleavage of pIgR, some amino acid residues remain associated with SC:immunoglobulin complexes but are eventually degaraded and/or removed from such complexes (Ahnen et al., J. Clin. Invest. 77:1841-1848, 1986). According to the definiton of the secretory component used herein, such amino acids are not part of the SC. In certain embodiments of the invention, pIgR-targeting elements that do not recognize or bind to the SC are preferred.

Another way in which different portions of a pIgR molecule can be delineated is by reference to the domains thereof. A protein "domain" is a relatively small (i.e., < about 150 amino acids) globular unit that is part of a protein. A protein may comprise two or more domains that are linked by relatively flexible stretches of amino acids. In addition to having a semi-independent structure, a given domain may be largely or wholly responsible for carrying out functions that are normally carried out by the intact protein. In addition to domains that have been determined by in vitro manipulations of protein molecules, it is understood in the art that a "domain" may also have been identified in silico, i.e, by software designed to analyze the amino acid sequences encoded by a nucleic acid in order to predict the limits of domains. The latter type of domain is more accurately called a "predicted" or "putative" domain but, in the present disclosure, the term domain encompasses both known and predicted domains unless stated otherwise.

Extracellular domains 1 through 6 of pIgR molecules from several species are indicated in Figure 3 of Piskurich et al. (J. Immunol. 154:1735-1747, 1995). In rabbit pIgR, domains 2 and 3 are encoded by a single exon that is sometimes deleted by alternative splicing. A transmembrane domain is also present in pIgR, as is an intracellular domain. The intracellular domain contains signals for transcytosis and endocytosis. Domains of a pIgR molecule that are of particular interest in the present disclosure include but are not limited to domain 5, domain 6, the transmembrane domain and the intracellular domain.

Another way in which different portions of a pIgR molecule can be defined is by reference to amino acid sequences that are conserved between pIgR homologs (i.e, pIgR molecules isolated from non-human species; see below). Non-limiting examples of conserved amino acid sequences include those found in Table 2; see also Figure 2. (For brevity's sake, the one letter abbreviations for amino acids is used in Table 2, but a version of each sequence that employs the three letter amino acid designations may be found in the Sequence Listing; see also Table 1.)

Table 1: Abbreviations for Amino Acids

| Amino acid | One-letter Abbreviation | One-letter symbol |
|---|---|---|
| Alanine | Ala | A |
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartic Acid | Asp | D |
| Cysteine | Cys | C |
| Glutamine | Gln | Q |
| Glutamic acid | Glu | E |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Ile | I |
| Leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |
| Valine | Val | V |

Table 2: Amino Acid Sequences that are Conserved in pIgR Homologs

| Amino Acid Sequence Conserved among pIgR Homologs | Position of Amino Acid Residues in Human pIgR Relative to Amino Terminal Methionine* | SEQ ID NO: |
|---|---|---|
| LRKED | 297-301, inclusive | |
| QLFVNEE | 325-331, inclusive | |
| LNQLT | 410-414, inclusive | |

| | | |
|---|---|---|
| YWCKW | 476-480, inclusive | |
| GWYWC | 522-526, inclusive | |
| STLVPL | 624-629, inclusive | |
| SYRTD | 658-662, inclusive | |
| KRSSK | 732-737, inclusive | |

* As described in Figure 3 of Mostov and Kaetzel, Chapter 12 in: Mucosal Immunology, Academic Press, 1999, pages 181-211.

Thus, for example, a specific internal portion of a given pIgR molecule might be defined as a region that has an amino-terminal border that has the amino acid sequence EKYWCKW and a carboxy-terminal border having the amino acid sequence side having the amino acid sequence DEGWYWCG. In human pIgR, the region so defined would be the amino acid sequence of residues 474 through 529. In the present invention, regions of any given pIgR molecule that are of particular interest include but are not limited to the following regions that are not conserved between pIgR homologs from different species:

R1 From KRSSK to the carboxy terminus,
R2a From SYRTD to the carboxy terminus,
R2b From SYRTD to KRSSK,
R3a From STLVPL to the carboxy terminus,
R3b From STLVPL to KRSSK,
R3c From STLVPL to SYRTD,
R4a From GWYWC to the carboxy terminus,
R4b From GWYWC to KRSSK,
R4c From GWYWC to SYRTD,
R4d From GWYWC to STLVPL,
R5a From YWCKW to the carboxy terminus,
R5b From YWCKW to KRSSK,
R5c From YWCKW to SYRTD,
R5d From YWCKW to STLVPL,

R5e From YWCKW to GWYWC,

R6a From LNQLT to the carboxy terminus,

R6b From LNQLT to KRSSK,

R6c From LNQLT to SYRTD,

R6d From LNQLT to STLVPL,

R6e From LNQLT to GWYWC,

R6f From LNQLT to YWCKW,

R7a From QLFVNEE to the carboxy terminus,

R7b From QLFVNEE to KRSSK,

R7c From QLFVNEE to SYRTD,

R7d From LNQLT to STLVPL,

R7e From QLFVNEE to GWYWC,

R7f From QLFVNEE to YWCKW,

R7g From QLFVNEE to LNQLT,

R8a From LRKED to the carboxy terminus,

R8b From LRKED to KRSSK,

R8c From LRKED to SYRTD,

R8d From LRKED to STLVPL,

R8e From LRKED to GWYWC,

R8f From LRKED to YWCKW,

R8g From LRKED to LNQLT, and

R8h From LRKED to QLFVNEE.

Homologs of pIgR are also within the scope of the invention. Homologs of pIgR are pIgR proteins from species other than Homo sapiens. By way of non-limiting example, pIgR proteins from various species include those from humans, the rat, mouse, rabbit, cow and possum (Table 3). See also Figure 3 in Mostov and Kaetzel, Chapter 12, "Immunoglobulin Transport and the Polymeric Immunoglobulin Receptor" in Mucosal Immunity, Academic Press, 1999, pages 181-211; and Piskurich et al., J. Immunol. 154:1735-1747, 1995).

Table 3: pIgR and pIgR-like Proteins From Non-Human Species

| Organism | Accession Number(s) |
|---|---|
| Zebrafish (Brachydanio rerio) | 9863256, 8713834, 8282255, & 7282118 |
| Mouse (Mus musculus) | 8099664, 2804245, 6997240, 4585867, 4585866, 2688814, 2688813, 2688812, 2688811, 2688810, 2688809, 2688808, 2688807, 3097245, 3046754, 3046752, 3046751, 3046756, 3046755, 3046750, 3046748, 3046747 and 2247711 |
| Rat (Rattas norvegicus) | 2222806, 475572, 475571, 473408, 603168 and 603167 |
| Cow (Bos taurus) | 388279 |
| Possum (Trichosuras vulpecula) | 5305520, 5305518, 5305514 and 5305512 |

Also within the scope of the invention are pIgR-like proteins. A "pIgR-like protein" is a protein that has an amino acid sequence having homolgy to a known pIgR protein. In many instances, the amino acid sequences of such pIgR-like molecules have been generated by the in silico translation of a nucleic acid, wherein the nucleotide sequence of the nucleic acid has been determined but is not known to encode a protein. By way of non-limiting example, pIgR-like proteins include PIGRL1 (U.S. Patent 6,114,515); a mouse gene having an exon similar to one of pIgR's (GenBank Accession No. 6826652); and human proteins translated in silico that have homology to pIgR proteins (GenBank Accession Nos. 1062747 and 1062741).

**Substantially Identical and Homologous pIgR Molecules**

As used herein, a "homolog" of a pIgR protein or a pIgR-like protein is a protein is an isoform or mutant of human pIgR, or a protein in a non-human species that either (i) is "identical" with or is "substantially identical" (determined as described below) to an amino acid sequence in human pIgR, or (ii) is encoded by a gene that is identical or substantially identical to the gene encoding human pIgR. Non-limiting examples of types of pIgR isoforms include isoforms of differing molecular weight that result from, e.g., alternate RNA splicing or proteolytic

cleavage; and isoforms having different post-translational modifications, such as glycosylation; and the like.

Two amino acid sequences are said to be "identical" if the two sequences, when aligned with each other, are exactly the same with no gaps, substitutions, insertions or deletions. Two amino acid sequences are defined as being "substantially identical" if, when aligned with each other, (i) no more than 30%, preferably 20%, most preferably 15% or 10%, of the identities of the amino acid residues vary between the two sequences; or (ii) the number of gaps between or insertions in, deletions of and subsitutions of, is no more than 10%, preferably 5%, of the number of amino acid residues that occur over the length of the shortest of two aligned sequences. The entire amino acid sequence of two proteins may be substantially identical to one another, or sequences within proteins may demonstrate identity or substantial identity with sequences of similar length in other proteins. In either case, such proteins are substantially identical to each other. Typically, stretches of identical or substantially identical sequences occur over 5 to 25, preferably 6 to 15, and most preferably 7 to 10, nucleotides or amino acids.

One indication that nucleotide sequences encoding pIgR proteins are substantially identical is if two nucleic acid molecules hybridize to each other under stringent conditions. Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is about 0.02 M at pH 7 and the temperature is at least about 60°C.

Another way by which it can be determined if two sequences are substantially identical is by using an appropriate algorithm to determine if the above-described critera for substantially identical sequences are met. Sequence comparisons between two (or more) polynucleotides or polypeptides are typically performed by algorithms such as, for example, the local homology algorithm of Smith and Waterman (Adv. Appl. Math. 2:482, 1981), by the homology alignment algorithm of Needleman and Wunsch (J. Mol. Biol. 48:443, 1970), by the search for similarity method of Pearson

and Lipman (Proc. Natl. Acad. Sci. U.S.A. 85:2444, 1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis.), or by visual inspection.

**pIgR Ligands**

As used herein, the terms "ligand" and "targeting element" are synonymous and encompasses any type of molecule that is capable of binding to a preselected target molecule. A ligand may, by way of non-limiting example, be a small molecule, a nucleic acid, a polypeptide, and derivatives and conjugates thereof. The terms "ligand" and "targeting element" encompass any type of molecule or moiety that is capable of binding to its target molecule, and a "pIgR ligand" or "pIgR targeting element" encompasses molecules and moieties that can bind pIgR or a fragment thereof. Preferred fragments are the pIgR stalk molecule and oligopeptides containing amino acid sequences from pIgR.

Any ligand that binds pIgR to an effective degree is identifiable using the compositions and methods of the invention. By way of non-limiting example, a pIgR ligand may be an antibody; a bacterial protein that binds pIgR; a polypeptide identified by various methods described herein; a nucleic acid such as an aptamer; a small molecule identified by various methods described herein; a derivative of any of the preceding ligands; or a conjugate or mixture of two or more of the preceding ligands or derivatives thereof. Preferred are polypeptides that can be incorporated into fusion proteins.

The binding of a ligand is target-specific in the sense that, although other molecules may be present in a mixture in which ligands and target molecules are contacted with each other, the ligand does not appreciably bind to other (non-target) molecules. It is recognized that the strength of binding between pIgR and a pIgR ligand, i.e., the affinity of a pIgR ligand for pIgR, is a matter of degree. As used herein, "target-specific" means that the pIgR ligand has a stronger affinity for its target molecule (pIgR) than for contaminating molecules, and this difference in affinity is sufficient for a given aspect of the invention. In general, the target specificity of a pIgR ligand for pIgR is comparable to the specificity of antibodies for

their antigens. Thus, by way of non-limiting example, the specificity for a ligand for pIgR should be at least approximately that of a single chain antibody (sFv) for pIgR. Examples of sFv's that can be used to evaluate the target specificity of a pIgR ligand include but are not limited to sFv-5A and derivatives thereof, such as sFv-5AF, which bind to the stalk of pIgR and are described herein; and sFv's that bind to the secretory component (SC) such as, e.g., those described in U.S. Patent 6,072,041.

The specificity of the binding is defined in terms of the values of absolute and relative binding parameters, such as the comparative dissociation constants (Kd) of a ligand for its target molecule as compared to the dissociation constant with respect to the ligand and unrelated molecules and compositions. Typically, the Kd of a ligand with respect to its target molecule will be 2-fold, preferably 5-fold, more preferably 10-fold less, than the Kd of the ligand for unrelated molecules and compositions. Even more preferably the Kd will be 50-fold less, more preferably 100-fold less, and more preferably 200-fold less.

The binding affinity of the ligands with respect to target molecules is defined in terms of the dissociation constant (Kd). The value of Kd can be determined directly by well-known methods, and can be computed even for complex mixtures by methods such as those, for example, set forth in Caceci, M., et al., Byte (1984) 9:340-362. In some situations, direct determination of Kd is problematic and can lead to misleadingly results. Under such circumstances, a competitive binding assay can be conducted to compare the affinity of a ligand for its target molecule with the affinity of molecules known to bind the target molecule. The value of the concentration at which 50% inhibition occurs (Ki) is, under ideal conditions, roughly equivalent to Kd. Moreover, Ki cannot be less than Kd; determination of Ki sets a maximal value for the value of Kd. Under circumstances where technical difficulties preclude accurate measurement of Kd, measurement of Ki can conveniently be substituted to provide, at the very least, an upper limit for Kd.

Kd may be measured in solution using techniques and compositions described in the following publications. Blake, D.A.; Blake, R.C.; Khosraviani, M.; Pavlov, A.R. "Immunoassays for Metal Ions." Analytica Chimica Acta 1998, 376, 13-19. Blake, D.A.; Chakrabarti, P.; Khosraviani, M.; Hatcher, F.M.; Westhoff, C.M.; Goebel, P.; Wylie, D.E.; Blake, R.C. "Metal Binding Properties of a Monoclonal

Antibody Directed toward Metal-Chelate Complexes." Journal of Biological Chemistry 1996, 271(44), 27677-27685. Blake, D.A.; Khosraviani, M.; Pavlov, A.R.; Blake, R.C. "Characterization of a Metal-Specific Monoclonal Antibody." Aga, D.S.; Thurman, E.M., Eds.; ACS Symposium Series 657; American Chemical Society: Washington, DC, 1997; pp 49-60.

Kd is measured using immobilized binding components on a chip, for example, on a BIAcore chip using surface plasmon resonance. Surface plasmon resonance is used to characterize the microscopic association and dissociation constants of reaction between sFv directed against pIgG associated molecules and pIgR and pIgR fragments. Such general methods are described in the following references and are incorporated herein by reference (Vely F. Trautmann A. Vivier E., BIAcore analysis to test phosphopeptide-SH2 domain interactions, Methods in Molecular Biology. 121:313-21, 2000; Liparoto SF. Ciardelli TL., Biosensor analysis of the interleukin-2 receptor complex, Journal of Molecular Recognition. 12:316-21, 1999; Lipschultz CA. Li Y. Smith-Gill S., Experimental design for analysis of complex kinetics using surface plasmon resonance, Methods. 20):310-8, 2000; Malmqvist M., BIACORE: an affinity biosensor system for characterization of biomolecular interactions, Biochemical Society Transactions. 27:335-40, 1999; Alfthan K., Surface plasmon resonance biosensors as a tool in antibody engineering, Biosensors & Bioelectronics. 13:653-63, 1998; Fivash M. Towler EM. Fisher RJ., BIAcore for macromolecular interaction, Current Opinion in Biotechnology. 9:97-101, 1998; Price MR. Rye PD. Petrakou E. Murray A. Brady K. Imai S. Haga S. Kiyozuka Y. Schol D. Meulenbroek MF. Snijdewint FG. Von Mensdorff-Pouilly S. Verstraeten RA. Kenemans P. Blockzjil A. Nilsson K. Nilsson O. Reddish M. Suresh MR. Koganty RR. Fortier S. Baronic L. Berg A. Longenecker MB. Hilgers J. et al.; Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. San Diego, Calif., November 17-23, 1996, Tumour Biology. 19 Suppl 1:1-20, 1998; Malmqvist M. Karlsson R, Biomolecular interaction analysis: affinity biosensor technologies for functional analysis of proteins, Current Opinion in Chemical Biology. 1:378-83, 1997; O'Shannessy DJ. Winzor DJ., Interpretation of deviations from pseudo-first-order kinetic behavior in the characterization of ligand binding by biosensor technology, Analytical Biochemistry.

236:275-83, 1996; Malmborg AC. Borrebaeck CA, BIAcore as a tool in antibody engineering, Journal of Immunological Methods. 183:7-13, 1995; Van Regenmortel MH., Use of biosensors to characterize recombinant proteins, Developments in Biological Standardization. 83:143-51, 1994; O'Shannessy DJ., Determination of kinetic rate and equilibrium binding constants for macromolecular interactions: a critique of the surface plasmon resonance literature, Current Opinions in Biotechnology. 5:65-71, 1994). Additionally or alternatively, binding constants and kinetic constants are estimated using calorimetry, equilibrium dialysis, and stopped flow methods using absorbance, fluorsescence, light scattering, turbidity, fluorescence anisotropy, and the like.

**pIgR Binding Assays**

The ability of a pIgR ligand of the invention to bind different pIgR molecules, fragments and derivaties thereof, and to undergo endocytosis, transcytosis, and/or exocytosis is a desirable attribute of these proteins. The pIgR-binding capacity of fusion proteins are examined using the following techniques. Such assays include the following:

**Ex Vivo Testing of Ligand Binding**

The ex vivo pIgR binding capacity of a pIgR-targeted protein is assessed by measuring endocytosis or transcytosis of bound ligand in mammalian epithelial cells. Receptor-mediated endocytosis provides an efficient means of causing a cell to ingest material which binds to a cell surface receptor. (See Wu et al., J. Biol. Chem. 262:4429-4432, 1987; Wagner et al., Proc. Natl. Acad. Sci. USA 87:3410-3414, 1990, and published EPO patent application EP-A1 0388758). Any number of well known methods for assaying endocytosis may be used to assess binding. For example, binding, transcytosis, and internalization assays are described at length in Breiftfeld et al. (J. Cell Biol. 109:475-486, 1989).

Ligand-pIgR binding is measured by a variety of techniques known in the art, e.g., immunoasays and immunoprecipitation. By way of example, antibodies to the biologically active portion of a protein conjugate can be used to bind and precipitate detectably labeled pIgR molecules; the amount of labeled material that is precipitated

corresponds to the degree of pIgR binding to a ligand such as, e.g., a protein conjugate having a pIgR-targeting element (see Tajima, J. Oral Sci. 42:27-31, 2000).

Apical Endocytosis is conveniently measured by binding a ligand such as a Fab fragment to the stalk at the apical surface of Madin-Darby canine kidney (MDCK) cells at 4°C, warming to 37°C. for brief periods (0-10 min), and cooling the cells back down to 4°C. Methods of pIgR expression in MDCK cells are known in the art (Breitfeld et al., Methods in Cell Biology 32:329-337, 01989). Fab remaining on the surface are removed by stripping at pH 2.3. Intracellular Fab molecules are those that remain cell-associated after the stripping, while surface-bound Fab are those removed by the acid wash. Controls for non-specific sticking include using pre-immune Fab and/or MDCK cells that are not transfected with pIgR.

Apical to Basolateral ("Reverse") Transcytosis is assessed by allowing MDCK cells to bind the Fab at the apical surface at 4°C, warming up to 37° for 0 to 240 min, and then measuring the amount of Fab delivered into the basolateral medium. This basolaterally-delivered Fab is compared to the sum of Fab that remains associated with the cells (intracellular or acid-stripped) and the Fab released back into the apical medium. Alternatively, transcystosis is assessed by continuously exposing cells to the Fab in the apical medium and measuring accumulation of Fab in the basolateral medium. This method avoids cooling the cells, but does not provide the kinetics of transporting a single cohort of ligand. In both methods degradation of the Fab can be assessed by running aliquots of the transcytosed Fab on SDS-PAGE and probing a Western with antibodies.

Basolateral Endocytosis is assessed by methods such as those described by Tajima (J. Oral Sci. 42:27-31, 2000). Non-specific transport (e.g. due to fluid phase endocytosis and transcytosis, or paracellular leakage between cells) can be controlled for by using MDCK cells that are not transfected with the pIgR and/or pre-immune Fab.

**Testing of Ligand Binding In vivo**

In vivo Transcytosis is assessed using pathogen-free experimental animals such as Sprague-Dawley rats. Detectably labeled ligand (e.g., a radioiodinated antibody) is administered into, e.g., the nares (the pair of openings of the nose or

nasal cavity of a vertebrate) or the intestine (see Example __). As will be understood by those of skill in the art, a "detectable label" is a composition or moiety that is detectable by spectroscopic, photochemical, biochemical, immunochemical, electromagnetic, radiochemical, or chemical means such as fluorescence, chemifluoresence, or chemiluminescence, or any other appropriate means

In vivo Apical to Basolateral ("Reverse") Transcytosis is assessed by measuring the delivery of a pIgR-targeting protein into the circulation as measured by the presence of a detectable label that has been incorporated into the protein that is being tested. The integrity of the ligand recovered from the circulation can be assessed by analyzing the ligand on SDS polyacrylamide gel electrophoresis.

## II. FUSION PROTEINS

A "protein" is a molecule having a sequence of amino acids that are linked to each other in a linear molecule by peptide bonds. The term protein refers to a polypeptide that is isolated from a natural source, or produced from an isolated cDNA using recombinant DNA technology; and has a sequence of amino acids having a length of at least about 200 amino acids.

A "fusion protein" is a type of recombinant protein that has an amino acid sequence that results from the linkage of the amino acid sequences of two or more normally separate polypeptides. Methods of preparing and using fusion proteins are described in U.S. patent application Serial No. 60/237,929 (attorney docket Nos. 030854.0009 and 057220-0301, entitled "Genetic Fusions of pIgR Ligands and Biologically Active Polypeptides for the Delivery of Therapeutic and Diagnostic Proteins" by Houston, L.L., Glynn, Jacqueline M., and Sheridan, Philip L.), filed October 2, 2000, which is incorporated in its entirety herein.

Proteins are polypeptides, i.e., polymers of structural units known as amino acids such as, e.g., proline (Pro), lysine (Lys), serine (Ser), alanine (Ala), methionine (Met) and the like. There are about twenty different types of amino acids that can be independently selected and arranged in any order to form a given protein. The structures of proteins are typically indicated by their amino acid sequences written from one end of the protein (the amino or "N" terminus) to the other end (the

carboxy or "C" terminus). Thus, an amino acid sequence of a protein is, for example, represented by:

[N terminus]-Met-Pro-Lys-Ala-Ala-Pro-Ser-Pro-Ser-Met-Ser-Ala-[C-terminus]

Proteins typically carry out or regulate specific biological functions in their natural setting, i.e, in vivo. Molecular biology has provided the tools necessary for the delivery of nucleic acids that encode therapeutic proteins, i.e., gene therapy agents (GTAs). Such proteins have therapeutic potential based on, e.g., their ability to compensate for the lack of functionality of a protein in diseased individuals. Insulin is the protodigm for such "protein replacement therapies."

Another way in which proteins, whether administered as purified proteins or as polypeptides encoded by GTAs, can act as therapeutic agents is by increasing (relative to the endogenous concentration) the concentration of a biologically active protein or compound that is normally present in biologically effective amounts in only certain areas of the body or during particular times. The increased availability of the protein causes its effects to reach tissues and cells that normally do not contact a biologically effective amount of the protein.

Another type of protein-based or gene therapy is based on proteins having cytotoxic, cytocidal, cytostatic properties or are capable of stimulating cellular growth. Cellular growth factors such as EGF (epidermal growth factor), NGF (neuron growth factor) and interleukins are examples of such proteins. Cytokines, which stimulate growth of cells in the immune system, are other examples of this type of therapeutic protein.

Another type of protein-based or gene therapy involves the production of polypeptide ligands, such as antibodies (described in more detail below), that bind to an endogenous molecule and thereby influence the activity or availability thereof. GTAs encoding other proteins, such as polypeptide ligands that bind cellular factors (including, e g., non-catalyzable versions of proteins that are substrates for intracellular enzymes and soluble receptor fragments), are also within the scope of the invention.

Therapies based on the production of mutant proteins and polypeptides are also within the scope of the invention. Mutant proteins and polypeptides have amino acid sequences that differ at one or more positions from the sequences of the natural (wildtype) protein. Due to the changes in its amino acid sequence, mutant proteins have altered or additional attributes that are not possessed by the wildtype protein.

One or more of the biological activities of a protein may be possessed by a biologically active polypeptide derived from the protein. A "polypeptide" is a polymer of amino acids that has an amino acid sequence that is, or is derived from, the complete (a.k.a. "full-length") amino acid sequence of a protein. By "biologically active" it is meant that the polypeptide itself has a biological effect, or that it causes, promotes, enhances, blocks, reduces, limits or modifies the production or activity of an endogenous molecule that has a biological effect. A "biological effect" is one that alters a process in an animal, or in a parasite of an animal, with a desired effect upon the animal. Desired effects include, by way of non-limiting example, preventing, treating or curing a disease or condition; killing or limiting the growth of a pathogen; or augmenting the phenotype or genotype of an animal.

Several characteristics of proteins and GTAs have limited their therapeutic application. These therapeutic agents are generally labile in general and in vivo in particular due to the proteases and nucleases found in various tissues, cells and fluids; and due to chemically or physically demanding environments, such as the acidic environment found in the stomach. Moreover, proteins are generally too large to pass epithelial cell barriers, which line the interiors of vessels in the pulmonary system and the gastrointestinal tract; the nasal and buccal cavities; the rectum and the vagina. Epithelial cell barriers also found on ocular and dermal surfaces. The present invention provides fusion proteins designed to be used in membrane-bounded vesicles, such as liposomes and viral envelope proteins, as well as viral structural (e.g., capsid) proteins.

As used herein, the term "polypeptide" encompasses polypeptides that are isolated molecular entities as well as polypeptides that are incorporated into the amino acid sequence of a larger polypeptide or protein. A protein that comprises polypeptides derived from two or more normally separate proteins is a "fusion protein." The present invention is drawn to fusion proteins that comprise a

transmembrane domain and a pIgR targeting element, or a pIgR ligand and a viral structural protein.

The invention thus provides compositions and methods for the delivery of proteins and GTAs into and through epithelial cells. Such epithelial cells line the interior of the gut and thus present the first obstacle that drugs must overcome as they are delivered from the interior of the gastrointestinal tract to other cells and fluids of the body. The invention thus provides for the delivery of biologically active purified proteins and GTAs via oral routes of administration, that is, via ingestion of a pill, capsule, tablet, or other encapsulated composition, as well as by other routes of gastrointestinal administration, e.g., via administration of a suppository. Because other epithelial cells line the airways of the lung, the invention also provides for the delivery of biologically active polypeptides via inhalation thereof.

A protein "domain" is a relatively small (i.e, < about 150 amino acids) globular unit that is part of a protein. A protein may comprise two or more domains that are linked by relatively flexible stretches of amino acids. In addition to having a semi-independent structure, a given domain may be largely or wholly responsible for carrying out functions that are normally carried out by the intact protein. In addition to domains that have been determined by in vitro manipulations of protein molecules, it is understood in the art that a "domain" may also have been identified in silico, i.e, by software designed to analyze the amino acid sequences encoded by a nucleic acid in order to predict the limits of domains. The latter type of domain is more accurately called a "predicted" or "putative" domain but, in the present disclosure, the term domain encompasses both known and predicted domains unless stated otherwise.

Proteins are encoded by nucleic acids known as DNA and RNA (deoxyribonucleic scid and ribonucleic acid, respectively). The nucleotide sequence of a nucleic acid contains the "blueprints" for a protein. Nucleic acids are polymers of nucleotides, four types of which are present in a given nucleic acid. The nucleotides in DNA are adenine, cytosine and guanine and thymine, (represented by A, C, G, and T respectively); in RNA, thymine (T) is replaced by uracil (U). The structures of nucleic acids are represented by the sequence of its nucleotides arranged in a 5′ ("5 prime") to 3′ ("3 prime") direction, e.g.,

5´-A-T-G-C-C-T-A-A-A-G-C-C-G-C-T-C-C-C-T-C-A-3´

In biological systems, proteins are typically produced in the following manner. A DNA molecule that has a nucleotide sequence that encodes the amino acid sequence of a protein is used as a template to guide the production of a messenger RNA (mRNA) that also encodes the protein; this process is known as transcription. In a subsequent process called translation, the mRNA is "read" and directs the synthesis of a protein having a particular amino acid sequence.

Each amino acid in a protein is encoded by a series of three contiguous nucleotides, each of which is known as a codon. In the "genetic code," some amino acids are encoded by several codons, each codon having a different sequence; whereas other amino acids are encoded by only one codon sequence. An entire protein (i.e., a complete amino acid sequence) is encoded by a nucleic acid sequence called a reading frame. A reading frame is a continuous nucleotide sequence that encodes the amino acid sequence of a protein; the boundaries of a reading frame are defined by its initiation (start) and termination (stop) codons.

The process by which a protein is produced from a nucleic acid can be diagrammed as follows:

DNA (A-T-G) - (A-A-G) - (C-C-G) - (C-T-C) - (C-C-T) - ... (etc.)

⇓ Transcription

RNA (A-U-G) - (A-A-G) - (C-C-G) - (C-U-C) - (C-C-U) - ... (etc.)

⇓ Translation

Protein Met - Pro - Lys - Ala - Ala - ... (etc.)

A chimeric reading frame encoding a fusion protein is prepared as follows. A "chimeric reading frame" is a genetically engineered reading frame that results from the fusion of two or more normally distinct reading frames, or fragments thereof, each of which normally encodes a separate polypeptide. Using recombinant DNA techniques, a first reading frame that encodes a first amino acid sequence is linked to

a second reading frame that encodes a second amino acid sequence in order to generate a chimeric reading frame. Chimeric reading frames may also include nucleotide sequences that encode optional fusion protein elements (see below). A hypothetical example of a chimeric reading frame created from two normally separate reading frames is depicted in the following flowchart.

A first Reading Frame and "Protein-1":

DNA-1 (A-T-G) - (A-A-G) - (C-C-G) - (C-T-C) - (C-C-T) - ...
(etc.)

⇓ Transcription

RNA-1 (A-U-G) - (A-A-G) - (C-C-G) - (C-U-C) - (C-C-U) - ...
(etc.)

⇓ Translation

Protein-1 Met - Pro - Lys - Ala - Ala - ...
(etc.)

A second Reading Frame and "Protein-2":

DNA-2 ***(T-G-G)-(G-T-T)-(A-C-T)-(C-A-C)-(T-C-A)- ...***
***(etc.)***

⇓ Transcription

RNA-2 ***(U-G-G)-(G-U-U)-(A-C-U)-(C-A-C)-(U-C-A)- ...***
***(etc.)***

⇓ Translation

Protein-2 ***Trp - Val - Thr - His - Ser - ...***
***(etc.)***

A chimeric Reading Frame that encodes a Fusion Protein that has sequences from Protein-1 and Protein-2:

DNA-Chimera (A-T-G) - (A-A-G) - (C-C-G) - ***(C-A-C)-(T-C-A)- ...***
***(etc.)***

⇓ Transcription

RNA-Chimera (A-U-G) - (A-A-G) - (C-C-G) - ***(C-A-C)-(U-C-A)- ...***
***(etc.)***

⇓ Translation

Fusion Protein Met - Pro - Lys - ***His - Ser*** - ...
***(etc.)***

In order for a chimeric reading frame to be functional, each normally distinct reading frame therein must be fused to all of the other normally distinct reading frames in a manner such that all of the reading frames are in frame with each other. By "in frame with each other" it is meant that, in a chimeric reading frame, a first nucleic acid having a first reading frame is covalently linked to a second nucleic acid having a second reading frame in such a manner that the two reading frames are "read" (translated) in register with each other. As a result, the chimeric reading frame encodes one extended amino acid sequence that includes the amino acid sequences encoded by each of the normally separate reading frames. A "fusion protein" is a single protein having amino acid sequences derived from two or more normally separate proteins, and which is encoded by a chimeric reading frame.

**Optional Fusion Protein Elements**

In addition to pIgR targeting elements and transmembrane or viral structural proteins, the fusion proteins of the invention may further comprise one or more optional fusion protein elements. Such non biologically active elements include, but are not limited to, the following. It is understood that a chimeric reading frame will include nucleotide sequences that encode such optional elements, and that these nucleotide sequences will be positioned so as to be in frame with the reading frame encoding the fusion protein. Optional fusion protein elements may be inserted between the pIgR-targeting element and the biologically active polypeptide, upstream or downstream (amino proximal and carboxy proximal, respectively) of these and other elements, or within the pIgR-targeting element and the biologically active polypeptide. A person skilled in the art will be able to determine which optional element(s) should be included in a fusion protein of the invention, and in what order, based on the desired method of production and/or intended use of the fusion protein.

Protein delivery elements are optional fusion protein elements that facilitate the uptake of a protein into cells but which are not pIgR targeting elements. The ETA (detoxified exotoxin a) protein delivery element is described in U.S. Patent No. 6,086,900 to Draper. The VP22 protein delivery element is derived from herpes simplex virus-1 and vectors containing sequences encoding the VP22 protein delivery

element are commercially available from Invitrogen (Carlsbad, CA; see also U.S. Patent No. 6,017,735 to Ohare et al.). The Tat protein delivery element is derived from the amino acid sequence of the Tat protein of human immunodeficiency virus (HIV). See U.S. Patents 5,804,604; 5,747,641; and 5,674,980.

Organellar delivery elements are optional fusion protein elements that direct a fusion protein into or out of a specific organelle or organelles. For example, the ricin A chain can be included in a fusion protein to mediate its delivery, or the delivery of a vesicle or virion comprising it, from the endosome into the cytosol. Endsomal processing can limit gene transfer by certain gene delivery vectors to certain tissues (Duan et al., J. Clin. Invest. 105:1573-1587, 2000). Additionally or alternatively, delivery elements for other organelles or subcellular spaces such as the nucleus, nucleolus, mitochondria, the Golgi apparatus, the endoplasmic reticulum (ER), the cytoplasm, etc. Mammalian expression constructs that incorporate organellar delivery elements are commercially available from Invitrogen (Carlsbad, CA: pShooter™ vectors). An H/KDEL (i.e, His /Lys-Asp-Glu-Leu sequence) may be incorporated into a fusion protein of the invention, preferably at the carboxy-terminus, in order to direct a fusion protein to the ER (see Andres et al., J. Biol. Chem. 266:14277-142782, 1991; and Pelham, Trends Bio. Sci. 15:483-486, 1990).

Another type of organellar delivery element is one which directs the fusion protein to the cell membrane and which may include a membrane anchoring element. Depending on the nature of the anchoring element, it can be cleaved on the internal or external leaflet of the membrane, thereby delivering the fusion protein to the intracellular or extracellular compartment, respectively. For example, it has been demonstrated that mammalian proteins can be linked to i) myristic acid by an amide-linkage to an N-terminal glycine residue, to ii) a fatty acid or diacylglycerol through an amide- or thioether-linkage of an N-terminal cysteine, respectively, or covalently to iii) a phophotidylinositol (PI) molecule through a C-terminal amino acid of a protein (for review, see Low, Biochem. J. 244:1-13, 1987). In the latter case, the PI molecule is linked to the C-terminus of the protein through an intervening glycan structure, and the PI then embeds itself into the phopholipid bilayer; hence the term "GPI" anchor. Specific examples of proteins know to have GPI anchors and their C-terminal amino acid sequences have been reported (see Table 1 and Figure 4 in Low,

Biochemica et Biophysica Acta, 988:427-454, 1989; and Table 3 in Ferguson, Ann. Rev. Biochem., 57:285-320, 1988). Incorporation of GPI anchors and other membrane-targeting elements into the amino- or carboxy-terminus of a fusion protein can direct the chimeric molecule to the cell surface.

Detectable polypeptides are optional fusion protein elements that either generate a detectable signal or are specifically recognized by a detectably labeled agent. An example of the former class of detectable polypeptide is green fluorescent protein (GFP). Examples of the latter class include epitopes such the "FLAG tag" and the c-myc epitope. These and other epitopes can be detected using labeled antibodies that are specific for the epitope; several such antibodies are commercially available.

Protein purification elements (a.k.a. protein isolation elements) are amino acid sequences that can be incorporated into a fusion protein in order to facilitate the purification or isolation of a fusion protein from a mixture containing other molecules.

Of particular interest are purification elements that can be used to isolate a fusion protein from the host cells or media of an expression system. Examples of purification elements include a "His tag" (6 contiguous His residues, a.k.a. 6xHis), which binds to surfaces that have been coated with nickel; streptavidin or avidin, which bind to surfaces that have been coated with biotin or "biotinylated" (see U.S. Patent 4,839,293 and Airenne et al., Protein Expr. Purif. 17:139-145, 1999); and glutathione-s-transferase(GST), which binds glutathione (Kaplan et al., Protein Sci. 6:399-406, 1997; U.S. Patent 5,654,176). Polypeptides that bind to lead ions have also been described (U.S. Patent 6,111,079). "Epitope tags" such as the c-myc epitope or FLAG-tag can be used to purify recombinant proteins via affinity chromatography using antibodies to such epitope tags.

Protein purification elements also include secretion sequences that direct recombinantly produced proteins out of the host cell and into the cellular media. Secreted proteins can be then be separated from the host cells that produce them by simply collecting the media. Examples of secretion elements include those described in U.S. Patents 5,846,818; 5,212,070; 5,631,144; 5,629,172; and 6,103,495; and Hardig et al., J. Biol. Chem. 268:3033-3036, 1993; Sizmann et al., Year Immunol.

7:119-130, 1993; and Power et al., Gene 113:95-99, 1992). Protein purification elements also include sequences that direct a recombinant protein to the periplasmic space of bacteria (Battistoni et al., Protein Expr. Purif. 6:579-587, 1995). Those skilled in the art will be able to determine which purification elements are desired, appropriate or necessary for a given fusion protein and/or expression system.

As used herein, the term "protein purification element" also includes elements designed to enhance the solubility and or assist in the proper folding of a protein. Such elements include GST and members of the 14-3-3 family of proteins (U.S. Patent 6,077,689).

**Spacers**

Spacers (a.k.a. linkers) are amino acid sequences that can be included in a fusion protein in between other portions of a fusion protein (e.g., between the biologically active polypeptide and the pIgR-targeting element, or between an optional fusion protein element and the remainder of the fusion protein). Spacers can be included for a variety of reasons. For example, a spacer can provide some physical separation between two parts of a protein that might otherwise interfere with each other via, e.g., steric hinderance. One example of a spacer of this type is the repeating amino acid sequence (Gly4-Ser)x, wherein x is 1 to 10, and preferably 1 to 4.

**"Rigid" Spacers**

A spacer may be designed to include two or more proline helices. For linkages between protein and polypeptide domains, a rigid structure can be provided at two or more positions to provide for the expression of biological function of each domain independently. For example, using a spacer with two or more proline helices, a sFv that recognizes pIgR stalk can be genetically linked to the transmembrane domain of another protein.

U.S. Patent No. 4,894,443 discloses the use of a spacer between a toxin and a cysteine. The sequence of the spacer is GTGSG(P)6SGSGTC, where G is glycine, T is threonine, S is serine, P is proline, and C is cysteine. A hexaproline sequence is known to form a helix (with a larger diameter than the normal alpha helix found in

proteins) that provides for the formation of a rigid structure (a rigid rod-like structure).

Non-limiting examples of relatively rigid spacers includes structures having the following formulae S1 and S2. In each instance, the length of proline sequences within a single spacer may be varied.

S1: $D1—[(Gly_{(b)}Ser)_x—Pro_{(a)}]_m—D2$

Where:

D1 and D2 are protein domains;

b is 1 to 6, preferably 4;

x is 0 to 4, preferably 1;

a is 6 to 1, preferably 6 to 8; and

m is 2 to 4, preferably 2.

S2: $D1—Pro_{(r)}—[(Gly_{(b)}Ser)_x—Pro_{(a)}]_m—Pro_{(s)}—D2$

Where:

domain 1- (Pro)r [- (GlyiSer)x - ]m (Pa)s - domain 2

where a is 6 to 12, preferably 6 to 8;

r, s, and x are independently 0 to 4, preferably 0 or 1;

r and s may not both be 0; and

m is 2 to 4, preferably 1 to 3.

Non-limiting examples of specific structures inlcude:

S3: $sFv-(G_jS)_x-P_a-(G_kS)_y-P_b-(G_iS)_z-hGH$

where

G is Glycine (Gly),

S is Serine (Ser),

P is Proline (Pro),

sFv is a single-chain antibody,

hGH is human growth hormone,

j is 1 to 6, preferably 4,

x, y and z are independently 1 to 4, preferably 1,

a and b are independently 6 to 12, preferably 6 to 8, and

k and l are independently 1 to 6, preferably 4.

**"Flexible" Spacers**

G4S is an example of a flexible linker and other residues may be subtituted that provide flexibility. SG4 is another example of a flexible linker. The same flexible linker does not need to be used in all possible positions. The side chains within this sequence should be relatively small compared to tyrosine, phenylalanine, and tryptophan. Side chains that may be substituted include alanine, threonine, and valine.

$sFv-(G_4S)_1-(P)_6-(G_4S)_1-(P)_6-(G_4S)_1-hGH$

The spacer region sequence (mRNA, SEQ ID NO:__ and protein, SEQ ID NO:__) is:

```
GGC GGU GGC GGU AGC CCG CCA CCG CCA CCG CCU GGU GGU GGC
GGC -
  G   G   G   G   S   P   P   P   P   P   P   G   G   G   G
-

- AGC CCG CCA CCG CCA CCU CCG CCG GGU GGC GGU GGC AGC
- S   P   P   P   P   P   P   P   G   G   G   G   S
```

**Protease Cleavage Sites**

In related embodiments of the invention, the pIgR-targeted fusion proteins can be designed so as to contain a site (a "protease cleavage site" or simply "cleavage site") that is amenable to being cleaved by agents or under conditions that cause or promote such cleavage. In some preferred embodiments of the invention, the cleavage site is contained within a spacer element, so that cleavage separates, e.g., the pIgR targeting element of a fusion protein from the biologically active polypeptide thereof, which is useful for in vivo therapeutic methods; or between an optional protein purification element and the remainder of the fusion protein, which is useful

for removing extraneous and potentially interfering purification elements in the process of purifying the fusion protein in vitro.

The nature and arrangement of a cleavage site or of a spacer containing a cleavage site will depend on the nature of the in vivo or in vitro method(s) of interest. It is understood by those skilled in the art that the amino acids sequences of fusion proteins that one wishes to have cleaved by a protease must be designed so as to retain the protease cleavage site of choice. Non-limiting examples of in vitro and in vivo cleavage sites and systems are as follows.

**In vivo cleavage.** Polypeptide fragments derived from the spacer and other optional fusion protein elements may be independently released from the cleaved fusion protein, or may remain associated with the pIgR targeting element or biologically active polypeptide. Most preferably, the cleavage reaction will predominantly occur after the fusion protein has been transported into or across an epithelial cell, or within a subcellular compartment, e.g., an organelle. For example, and for illustrative purposes only, the cleavage reaction might be effectuated by a protease or esterase found in an epithelial cell, by the acidic conditions found near a tumor cell, by conditions in the blood that destabilize disulfide conjugation, or by a protease found in an organelle.

Preferred cleavage sites for in vivo applications include but are not limited to those that are recognized by caspases, which can be used, e.g., to cleave and activate a biologically active polypeptide from a fusion protein during early events in apoptosis; proteases specific for an organelle into which it is desired to deliver a fusion protein, with one intended result being that a biologically active portion of the cleaved fusion protein will be retained by the organelle (i.e, organellar leader sequences).

Caspases are intracellular cysteine proteases which have been shown to play an essential role in the initiation and execution phases of apoptotic cell death. For reviews, see Fadeel et al. (IUBMB Life 49:421-425, 2000), Anderson (Cell Death Differ. 7:589-602, 2000) and Earnshaw et al., Annu. Rev. Biochem. 68:383-424, 1999). Fusion proteins can be designed so as to require proteolytic activation before it becomes biologically active. Inclusion of a given caspase cleavage site in such a fusion protein can be used to design fusion proteins that are cleaved by a particular

caspase is activated. In instances where the biologically active component of a fusion protein is not active until released from the fusion protein, the latter type of fusion proteins provide biologically polypeptides that act at specific times during the apoptotic process. Cathepsins may be used in the same way in other vesicular compartments of the cell.

Organellar leader sequences include, by way of non-limiting example, mitochondrial leader peptides that are proteolytically removed from proteins after their transport into mitochondria.

**In vitro cleavage**. Cleavable spacers may also be used for other purposes, especially in protein purification schemes. Consider, as an example, the case of a fusion protein that has an amino terminal 6xHis tag, and a protease cleavage site located immediately carboxy terminal from the His tag, i.e, between the His tag and the remainder of the fusion protein being produced. After the fusion protein has been purified using the His tag's affinity for Nickel-coated surfaces, it is then cleaved with the appropriate protease in order to separate the His tag from the remainder of the protein. It is often desirable to remove elements such as His tags that are useful for protein purification purposes but might interfere with the biological activity of the fusion proteins. Cleavable spacers may be designed so as to regenerate the amino terminal amino acid sequence present in the original protein.

Preferred cleavage sites for in vitro applications include but are not limited to those that recognize a cleavage site, which may be introduced into a fusion protein by genetic manipulation, that is located between a portion of the fusion protein that is not required for, and may even be detrimental to, the in vivo uses for which the fusion protein is intended. Commercially available expression systems that may be used to introduce cleavage sites include by way of non-limiting example cleavage sites that are recognized by enterokinase, trypsin, Factor Xa, Factor IXa and thrombin.

Enterokinase may be used to cleave spacer elements (see U.S. Patent 4,745,069). A preferred enterokinase is one that is produced via recombinant DNA techniques, as it is virtually free of other proteases and is able to efficiently cleave fusion proteins in partially purified preparations (Collins-Racie et al., Biotechnology 13:982-987, 1995). Moreover, enterokinase is relatively permissive regarding the amino acid residue downstream of the recognition sequence (Hosfield et al., Anal.

Bochem. 269:10-16, 1999). Trypsin may also be used in this fashion (U.S. Patent 6,037,143). In addition to providing cleavage sites for purification protein purposes, in vivo cleavage by gastrointestinal proteases such as enterokinase or trypsin may serve as a mechanism by which a fusion protein is released from a carrier in the gut.

Factor Xa (Peter et al., Circulation 101:1158-1164, 2000; U.S. Patent 6,010,883) and thrombin are blood coagulation factors. Expression vectors may comprise a sequence encoding a cleavage site for thrombin or Factor Xa that can be used to remove a purification element (such as a His tag) from the fusion protein after it has served its purification purpose.

**Post-Translational Modifications**

Different host cells will contain different post-translational modification mechanisms that may provide particular types of post-translational modification of a fusion protein if the amino acid sequences required for such modifications is present in the fusion protein. A large number ($\sim 100$) of post-translational modifications have been described, a few of which are discussed herein. One skilled in the art will be able to choose appropriate host cells, and design chimeric genes that encode fusion proteins comprising the amino acid sequence needed for a particular type of modification.

Glycosylation is one type of post-translational chemical modification that occurs in many eukaryotic systems, and may influence the activity, stability, pharmacogenetics, immunogenicity and/or antigenicity of proteins. However, specific amino acids must be present at such sites to recruit the appropriate glycosylation machinery, and not all host cells have the appropriate molecular machinery. Sacharomyces cerevisieae and Pichia pastoris provide for the production of glycosylated proteins, as do expression systems that utilize insect cells, although the pattern of glyscoylation may vary depending on which host cells are used to produce the fusion protein.

Another type of post-translation modification is the phosphorylation of a free hydroxyl group of the side chain of one or more Ser, Thr or Tyr residues. Protein kinases catalyze such reactions. Phosphorylation is often reversible due to the action

of a protein phosphatase, an enzyme that catalyzes the dephosphorylation of amino acid residues.

Differences in the chemical structure of amino terminal residues result from different host cells, each of which may have a different chemical version of the methionine residue encoded by a start codon, and these will result in amino termini with different chemical modifications.

For example, many or most bacterial proteins are synthesized with an amino terminal amino acid that is a modified form of methionine, i.e, N-formyl-methionine (fMet). Although the statement is often made that all bacterial proteins are synthesized with an fMET initiator amino acid; although this may be true for E. coli, recent studies have shown that it is not true in the case of other bacteria such as Pseudomonas aeruginosa (Newton et al., J. Biol. Chem. 274:22143-22146, 1999). In any event, in E. coli, the formyl group of fMet is usually enzymatically removed after translation to yield an amino terminal methionine residue, although the entire fMet residue is sometimes removed (see Hershey, Chapter 40, "Protein Synthesis" in: Escherichia Coli and Salmonella Typhimurium: Cellular and Molecular Biology, Neidhardt, Frederick C., Editor in Chief, American Society for Microbiology, Washington, D.C., 1987, Volume 1, pages 613-647, and references cited therein.) E. coli mutants that lack the enzymes (such as, e.g., formylase) that catalyze such post-translational modifications will produce proteins having an amino terminal fMet residue (Guillon et al., J. Bacteriol. 174:4294-4301, 1992).

In eukaryotes, acetylation of the initiator methionine residue, or the penultimate residue if the initiator methionine has been removed, typically occurs co- or post-translationally. The acetylation reactions are catalyzed by N-terminal acetyltransferases (NATs, a.k.a. N-alpha-acetyltransferases), whereas removal of the initiator methionine residue is catalyzed by methionine aminopeptidases (for reviews, see Bradshaw et al., Trends Biochem. Sci. 23:263-267, 1998; and Driessen et al., CRC Crit. Rev. Biochem. 18:281-325, 1985). Amino terminally acetylated proteins are said to be "N-acetylated," "N alpha acetylated" or simply "acetylated."

Another post-translational process that occurs in eukaryotes is the alpha-amidation of the carboxy terminus. For reviews, see Eipper et al. Annu. Rev. Physiol. 50:333-344, 1988 and Bradbury et al. Lung Cancer 14:239-251, 1996.

About 50% of known endocrine and neuroendocrine peptide hormones are alpha-amidated (Treston et al., Cell Growth Differ. 4:911-920, 1993). In most cases, carboxy alpha-amidation is required to activate these peptide hormones.

Because in vivo post-translational modifications result from systems within host cells, different expression systems will be needed to realize different modifications. One skilled in the art will be able to ascertain whether such modifications are necessary for the activity or stability of a given fusion protein, and will be able to choose appropriate expression systems and introduce into the fusion protein the appropriate chemical modification elements. Most proteins can be expressed in a variety of expression systems by those skilled in the art. For example, IL-2 proteins from various species have been cloned and expressed in E. coli (Devos et al., Nucleic Acids Res. 11:4307-4323, 1983), Streptomyces lividans (Munoz et al., Biochem. Biophys. Res. Commun. 133:511-519, 1985; S. cerevisiae (Zhang et al., Chung Kuo I Hsueh Ko Hsueh Pao 17:274-280, 1995), in insect cells (Kashima et al., J. Vet. Med. Sci. 61:705-707, 1999), and in transfected mammalian cells (Knezevic et al., Phlugers Arch. 431:R2-R228). Some of the above-described modifications can be made in vitro after the fusion protein has been expressed. For details, see U.S. Patent No. 5,656,456 to Stout et al.

**Recombinant DNA Expression Systems**

In order to achieve recombinant expression of a fusion protein, an expression cassette or construct capable of expressing a chimeric reading frame is introduced into an appropriate host cell to generate an expression system. The expression cassettes and constructs of the invention may be introduced into a recipient prokaryotic or eukaryotic cell either as a nonreplicating DNA or RNA molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the gene may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced DNA sequence into the host chromosome.

Host cells which may be used in the expression systems of the present invention are not strictly limited, provided that they are suitable for use in the

expression of the chimeric pIgR-targeting peptide of interest. Suitable hosts may often include eukaryotic cells. Preferred eukaryotic hosts include, for example, yeast, fungi, insect cells, mammalian cells either in vivo, or in tissue culture.

Expression cassettes and constructs may be introduced into an appropriate host cell by any of a variety of suitable means, i.e, transformation, transfection, conjugation, protoplast fusion, electroporation, particle gun technology, calcium phosphate-precipitation, direct microinjection, and the like. After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene(s) results in the production of a chimeric pIgR-targeting peptide of the invention, or fragments thereof. This can take place in the transformed cells as such, or following the induction of these cells to differentiate (for example, by administration of bromodeoxyuracil to neuroblastoma cells or the like). A variety of incubation conditions can be used to form the peptide of the present invention. The most preferred conditions are those which mimic physiological conditions.

The introduced nucleic acid molecule can be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

A variety of recombinant DNA expression systems may be used to produce the fusion proteins of the invention. Expression systems of particular interest include prokaryotic systems, yeast expression systems, insect expression systems mammalian expression systems.

Prokaryotic expression systems utilize plasmid and viral (bacteriophage) expression vectors that contain replication sites and control sequences derived from a species compatible with the host may be used. Suitable phage or bacteriophage vectors may include λgt10, λgt11 and the like; and suitable virus vectors may include pMAM-neo, pKRC and the like. Appropriate prokaryotic plasmid vectors include

those capable of replication in E. coli (such as, by way of non-limiting example, pBR322, pUC118, pUC119, ColEl, pSC101, pACYC 184, πVX; "Molecular Cloning: A Laboratory Manual", 1989, supra). Bacillus plasmids include pC194, pC221, pT127, and the like (Gryczan, In: The Molecular Biology of the Bacilli, Academic Press, NY, pp. 307-329, 1982). Suitable Streptomyces plasmids include p1J101 (Kendall et al., J. Bacteriol. 169:4177-4183, 1987), and streptomyces bacteriophages such as ϕC31 (Chater et al., In: Sixth International Symposium on Actinomycetales Biology, Akademiai Kaido, Budapest, Hungary, pp. 45-54, 1986). Pseudomonas plasmids are reviewed by John et al. (Rev. Infect. Dis. 8:693-704, 1986), and Izaki (Jpn. J. Bacteriol. 33:729-742, 1978). See also Brent et al., "Vectors Derived From Plasmids," Section II, and Lech et al. "Vectors derived from Lambda and Related Bacteriophages" Section III, in Chapter 1 of Short Protocols in Molecular Biology, 2nd Ed., Ausubel et al., eds., John Wiley and Sons, New York, 1992, pages 1-13 to 1-27; Lech et al. "Vectors derived from Labmda and Related Bacteriophages" Section III and Id. pages 1-28 to page 1-52.

Recognized prokaryotic hosts include bacteria such as E. coli, Bacillus, Streptomyces, Pseudomonas, Salmonella, Serratia, and the like. However, in such hosts, the fusion protein will not be glycosylated. In any event, the host cell must be compatible with the replicon and control sequences in the expression cassette.

To express a chimeric pIgR-targeting peptide of the invention (or a functional derivative thereof) in a prokaryotic cell, it is necessary to operably link the sequence encoding the chimeric pIgR-targeting peptide of the invention to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable (i.e, inducible or derepressible). Examples of constitutive promoters include the int promoter of bacteriophage λ, the bla promoter of the β-lactamase gene sequence of pBR322, and the cat promoter of the chloramphenicol acetyl transferase gene sequence of pPR325, and the like. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage λ (PL and PR), the trp, recA, λacZ, λacI, and gal promoters of E. coli, the α-amylase (Ulmanen et al., J. Bacteriol. 162:176-182, 1985) and the ς-28-specific promoters of B. subtilis (Gilman et al., Gene Sequence 32:11-20, 1984), the promoters of the bacteriophages of Bacillus (Gryczan, in: The Molecular Biology of the Bacilli,

Academic Press, Inc., NY, 1982), and Streptomyces promoters (Ward et al., Mol. Gen. Genet. 203:468-478, 1986). Prokaryotic promoters are reviewed by Glick (Ind. Microbiot. 1:277-282, 1987), Cenatiempo (Biochimie 68:505-516, 1986), and Gottesman (Ann. Rev. Genet. 18:415-442, 1984).

Proper expression in a prokaryotic cell also requires the presence of a ribosome-binding site upstream of the gene sequence-encoding sequence. Such ribosome-binding sites are disclosed, for example, by Gold et al. (Ann. Rev. Microbiol. 35:365-404, 1981). The selection of control sequences, expression vectors, transformation methods, and the like, are dependent on the type of host cell used to express the gene. As used herein, "cell", "cell line", and "cell culture" may be used interchangeably and all such designations include progeny. Thus, the words "transformants" or "transformed cells" include the primary subject cell and cultures derived therefrom, without regard to the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. However, as defined, mutant progeny have the same functionality as that of the originally transformed cell.

Bacterial systems may also be used to create and produce large amounts of shuttle vectors. Shuttle vectors are constructs designed to replicate in a prokaryotic host such as E. coli but which contain sequences that allow the shuttle vector and a chimeric reading frame incorporated therein to be transferred to a eukaryotic viral vector or other vector such as baculovirus or adenovirus.

Yeast expression systems can be utilized which incorporate promoter and termination elements from the actively expressed sequences coding for glycolytic enzymes that are produced in large quantities when yeast are grown in mediums rich in glucose. Known glycolytic gene sequences can also provide very efficient transcriptional control signals. Yeast cells provide a substantial advantage over prokarytoic expression systems in that they can carry out post-translational modifications of fusion proteins. A number of recombinant DNA strategies exist utilizing strong promoter sequences and high copy number plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian genes and secretes peptides bearing leader sequences (i.e, pre-peptides).

Preferred yeast expression vectors include those derived from the episomal element known as the 2-micron circle as well as derivatives of yeast integrating (YIp), yeast replicating (YRp), yeast centromeric (YCp), yeast episomal (YEp), and yeast linear (YLp) plasmids (Broach, in: The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470, 1981; Lundblad et al., Section II and, Becker et al., Section III, of Chapter 13 in: Short Protocols in Molecular Biology, 2nd Ed., Ausubel et al., eds., John Wiley and Sons, New York, 1992, pages 13-19 to 13-41).

Insect expression systems utilize insect host cells, such s, e.g., sf9 and sf21 cells, both of which are derived from the iplbsf-21 cell line derive from the pupal ovarian tissue of the fall army worm spodoptera frugiperda (O'Reilly et al., Baculovirus expression vectors: A Laboratory Manual New York, New York, W.H. Freeman and Company. See also baculovirus expression protocols in Methods in Molecular Biology Vol. 39; Richardson ed. Humana Totowa New Jersey, 1992; and Vaughn et al., In vitro 13:213-217, 1977. The cell line bti-tn-5b1-4 (high 5 tm cell line), which originated from the ovarian cells of the cabbage luper, Trichoplusa ni (Davis et al., Biotechnology 10:1148-1150, 1992; Granados et al., J.Invertebr. Pathol. 64:260-266, 1994; Wickham et al., Biotechnology Prog. 8:391-396, 1992; Wickham et al., Biotechnol. Prog. 9:25-30, 1993). Other insect cell lines that can be used to express baculo virus vectors have been described (Hink et al., Biotechnol. Prog. 7:9-14, 1991). See, also Piwnica-Worms "Expression of Proteins in Insect Cells Using Baculo Viral Vectors" section II in chapter 16 of Short Protocols in Molecular Biology, second edition, Ausubel et al, eds., John Wiley and Sons, New York, New York 1992. Using insect cells as hosts, the Drosophila alcohol dehydrogenase promoter can be used (Rubin, Science 240:1453-1459, 1988). Alternatively, baculovirus vectors can be engineered to express large amounts of chimeric pIgR-targeting peptides of the invention in insect cells (Jasny, Science 238:1653, 1987; Miller et al., in: Genetic Engineering, Vol. 8, Plenum, Setlow et al., eds., pp. 277-297, 1986).

Mammalian expression systems utilize host cells such as HeLa cells, cells of fibroblast origin such as VERO or CHO-K1, or cells of lymphoid origin and their derivatives. Preferred mammalian host cells include SP2/0 and J558L, as well as

neuroblastoma cell lines such as IMR 332, which may provide better capacities for correct post-translational processing.

Several expression vectors are available for the expression of chimeric pIgR-targeting peptides of the invention in a mammalian host. A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, cytomegalovirus, simian virus, or the like, where the regulatory signals are associated with a particular gene sequence which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, and the like, may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the gene sequences can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical (such as metabolite) regulation.

Preferred eukaryotic plasmids include, for example, BPV, vaccinia, SV40, 2-micron circle, and the like, or their derivatives. Such plasmids are well known in the art (Botstein et al., Miami Wntr. Symp. 19:265-274, 1982; Broach, in: The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470, 1981; Broach, Cell 28:203-204, 1982; Bollon et al., J. Clin. Hematol. Oncol. 10:39-48, 1980; Maniatis, In: Cell Biology: A Comprehensive Treatise, Vol. 3, Gene Sequence Expression, Academic Press, NY, pp. 563-608, 1980).

Expression of chimeric pIgR-targeting peptides of the invention in eukaryotic hosts requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include, for example, the promoter of the mouse metallothionein I gene sequence (Hamer et al., J. Mol. Appl. Gen. 1:273-288, 1982); the TK promoter of Herpes virus (McKnight, Cell 31:355-365, 1982); the SV40 early promoter (Benoist et al., Nature (London) 290:304-31, 1981); and the yeast gal4 gene sequence promoter (Johnston et al., Proc. Natl. Acad. Sci. (USA) 79:6971-6975, 1982; Silver et al., Proc. Natl. Acad. Sci. (USA) 81:5951-5955, 1984).

Translation of eukaryotic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence which encodes a chimeric pIgR-targeting peptide of the invention (or a functional derivative thereof) does not contain any intervening codons which are capable of encoding a methionine (i.e, AUG). The presence of such codons results either in the formation of a fusion protein (if the AUG codon is in the same reading frame as the chimeric pIgR-targeting peptide of the invention coding sequence) or a frame-shift mutation (if the AUG codon is not in the same reading frame as the chimeric pIgR-targeting peptide of the invention coding sequence).

**Methods of Isolating and Purifying Fusion Proteins**

After synthesis, it is preferred that a pIgR-targeting fusion protein isolated or purified, preferably substantially purified. By "isolated" it is meant that the fusion protein has been separated from any molecule that interferes with the biological activity or pIgR-targeting capacity thereof. As used herein the term "substantially purified" means at least about 95%, preferably at least about 99%, free of other components used to produce and/or modify the fusion protein. The term "purified" refers to a fusion protein that has been separated from at least about 50% of undesirable elements.

Optional fusion protein elements can be incorporated into the fusion protein and used during its purification and/or preparation. For example, as is discussed in more detail above, a fusion protein may include a protein purification element such as, for example, a "His tag" (His6). A His-tagged fusion protein can be isolated, or at least partially purified, from a composition that further comprises undesirable compounds by contacting the composition with a column of nickel-plated beads. The His-tagged fusion protein will bind to the nickel plating and will thus be retained in the column; undesirable compounds pass through the column. As is explained above in more detail, various methods may be used to remove the protein purification element from the fusion protein after such steps.

Purity can be assessed by any suitable method, including HPLC analysis and staining of gels through which an aliquot of the preparation containing the fusion

protein has been electrophoresed. Those practiced in the art will know what degree of isolation or purification is appropriate for a given application. For example, (in the U.S. at least) biologicals do not have to meet the same standard of purity for, e.g., a compound.

Post-translational modifications to a fusion protein may created in vitro or in vivo. Various chemical treatments can be used for in vitro modifications of pure or semi-pure proteins; whereas in vivo modifications result from the choice of expression system and host cells. Post-translational modifications include, by way of non-limiting example, glycosylation, cleavage, phophorylation, cross-linking, formation or reduction of disulfide bridges, and the like

During or after the purification process, it is often desirable to monitor both the amount and biological activity of the protein being purified. The amount of protein can be detected by using antibodies directed to the protein, or to an epitope introduced thereinto. In the latter case, many antibodies are commercially available for such epitopes. Additionally or alternatively, the fusion protein may comprise a detectable polypeptide by which the fusion protein may be monitored.

Some of the biological activities of a fusion protein will vary depending on the nature of the biologically active polypypetide(s) included therein, and assays specific for the biological activities of the parent proteins are used. The fusion proteins are also assayed for their ability to bind pIgR and undergo various forms of cellular trafficking. Assays for these and pIgR-related attributes are described herein and are applicable to the fusion proteins of the invention.

## III. GENE THERAPY

The delivery of nucleic acids to treat diseases or disorders is known as gene therapy (Kay et al., Gene Therapy, Proc. Natl. Acad. Sci. USA 94:12744-12746, 1997). It has been proposed to use gene therapy to treat genetic disorders as well as pathogenic diseases. For reviews, see Desnick et al., Gene Therapy for Genetic Diseases, Acta Paediatr. Jpn. 40:191-203, 1998; and Bunnell et al., Gene Therapy for Infectious Diseases, Clinical Microbiology Reviews 11:42-56, 1998).

Gene delivery systems use vectors that contain or are attached to therapeutic nucleic acids (see Figures 5 and 17). These vectors facilitate the uptake of the nucleic

acid into the cell and may additionally help direct the nucleic acid to a preferred site of action, e.g., the nucleus or cytoplasm (Wu et al., "Delivery Systems for Gene Therapy," Biotherapy 3:87-95, 1991).

Different gene delivery vectors vary with regards to various properties, and different properties are desirable depending on the intended use of such vectors. However, certain properties (for example, safety, ease of preparation, etc.) are generally desirable in most circumstances. One property that varies among vectors is the ability to infect dividing and/or non-dividing cells. In the case of adults, the cells of which are dividing slowly (compared to fetuses, infants, and youn children), vectors that can infect non-dividing cells are preferably targeted for long-term gene therapy. Many retroviral vectors have a limited capacity to infect non-dividing cells. However, a vector that infects non-dividing cells, or, even more preferably, dividing as well as non-dividing cells, has a greater chance of infecting and transforming the cells of an adult animal.

**Targeting of Gene Delivery Vectors**

In order to enhance the effectiveness of gene delivery vectors, it is desirable to target specific cells or tissues of interest (targeted cells or tissues, respectively). This increases the effective dose (the amount of therapeutic nucleic acid present in the targeted cells or tissues) and minimizes side-effects due to distribution of the therapeutic nucleic acid to other cells. For reviews, see Peng et al., "Viral Vector Targeting," Curr. Opin. Biotechnol. 10:454-457, 1999; Gunzburg et al., "Retroviral Vector Targeting for Gene Therapy," Cytokines Mol. Ther. 2:177-184, 1996.; Wickham, "Targeting Adenovirus," Gene Ther. 7:110-114, 2000; Dachs et al., "Targeting Gene Therapy to Cancer: A Review," Oncol. Res. 9:313-325, 1997; Curiel, "Strategies to Adapt Adenoviral Vectors for Targeted Delivery," Ann NY Acad. Sci. 886:158-171, 1999; Findeis et al., "Targeted Delivery of DNA for Gene Therapy via Receptors," Trends Biotechnol. 11:202-205, 1993.

Some targeting strategies make use of cellular receptors and their natural ligands in whole or in part. See, for example, Cristiano et al., "Strategies to Accomplish Gene Delivery Via the Receptor-Mediated Endocytosis Pathway," Cancer Gene Ther., Vol. 3, No. 1, pp. 49-57, Jan. - Feb. 1996.; S.C. Philips,

"Receptor-Mediated DNA Delivery Approaches to Human Gene Therapy," Biologicals, Vol. 23, No. 1, pp. 13-6, March 1995; Michael et al., "Strategies to Achieve Targeted Gene Delivery Via the Receptor-Mediated Endocytosis Pathway," Gene Ther., Vol. 1, No. 4, pp. 223-32, July 1994; Lin et al., "Antiangiogenic Gene Therapy Targeting The Endothelium-Specific Receptor Tyrosine Kinase Tie2," Proc. Natl. Acad. Sci., USA, Vol. 95, pp. 8829-8834, 1998. Sudimack et al., "Targeted Drug Delivery Via the Folate Receptor," Adv. Drug Deliv., pp. 147-62, March 2000; Fan et al., "Therapeutic Application of Anti-Growth Factor Receptor Antibodies," Curr. Opin. Oncol., Vol. 10, No. 1, pp. 67-73, January 1998; Wadhwa et al., "Receptor Mediated Glycotargeting," J. Drug Target, Vol. 3, No. 2, pp. 111-27, 1995; Perales et al., "An Evaluation of Receptor-Mediated Gene Transfer Using Synthetic DNA-Ligand Complexes," Eur. J. Biochem, Vol. 1, No 2, pp. 226, 255-66, December 1994; Smith et al., "Hepatocyte-Directed Gene Delivery by Receptor-Mediated Endocytosis," Semin Liver Dis., Vol. 19, No. 1, pp. 83-92, 1999.

Antibodies to surface antigens specific for a particular cell type may also be used as targeting elements. See, for example, Kuroki et al., "Specific Targeting Strategies of Cancer Gene Therapy Using a Single-Chain Variable Fragment (scFv) with a High Affinity for CEA," Anticancer Res., pp. 4067-71, 2000; U.S. Patent 6,146,885, to Dornburg, entitled "Cell-Type Specific Gene Transfer Using Retroviral Vectors Containing Antibody-Envelope Fusion Proteins"; Jiang et al., "In Vivo Cell Type-Specific Gene Delivery With Retroviral Vectors That Display Single Chain Antibodies," Gene Ther. 1999, 6:1982-7; Engelstadter et al., "Targeting Human T Cells By Retroviral Vectors Displaying Antibody Domains Selected From A Phage Display Library," Hum. Gene Ther. 2000, 11:293-303; Jiang et al., "Cell-Type-Specific Gene Transfer Into Human Cells With Retroviral Vectors That Display Single-Chain Antibodies," J. Virol 1998,72:10148-56; Chu et al., "Toward Highly Efficient Cell-Type-Specific Gene Transfer With Retroviral Vectors Displaying Single-Chain Antibodies," J. Virol 1997, 71:720-5; Chu et al., "Retroviral Vector Particles Displaying The Antigen-Binding Site Of An Antibody Enable Cell-Type-Specific Gene Transfer," J. Virol 1995, 69:2659-63; and Chu et al., "Cell Targeting With Retroviral Vector Particles Containing Antibody-Envelope Fusion Proteins," Gene Ther. 1994, 1:292-9.

A different strategy uses bispecific binding molecules (e.g., antibodies) to preferentially target gene delivery vectors to selected cell types. In brief, an antibody (or other binding molecule) that binds both the gene delivery vector and a protein (e.g., a receptor or surface antigen) preferentially displayed on the cells of interest. The bivalent binding molecule acts as a "bridge" between the cellular surface molecule and the gene delivery vector. See, e.g., U.S. Patent No. 5,712,136. Multivalent, including bivalent, pIgR-targeted proteins are described in U.S. patent application Serial No. 60/267,601, attorney docket No. 057220.0401, entitled "Polyspecific Binding Molecules Having a Polymeric Immunoglobulin Receptor Binding Region" by Houston, L.L., and Sheridan, Philip L., filed February 9, 2001.

Targeting strategies can be applied to virus-derived gene delivery vehicles (virions) as well as liposomes. However, different considerations are taken into account in different systems. For example, a targeting element that is derived from a viral capsid protein is likely to be inapplicable to liposomal formulations, but a transmembrane targeting element might be used in both liposomal and viral membranes (the latter being present in enveloped viruses).

**Targets for Gene Therapy**

A variety of diseases are potentially amenable to gene therapy (Table 4). Presently, gene therapy clinical trials are underway for Monogenic Diseases, such as X-linked severe combined immunodeficiency, ADA deficiency (ADA-SCID), Mucopolysaccharidosis, Familial hypercholesterolemia, Cystic fibrosis, Hemophilia B (factor IX deficiency), and Chronic granulomatous disease (p47phox deficiency); cancers, such as ovarian cancer, pulmonary carcinoma, head and neck cancer, non-small cell lung cancer, hematologic malignancies, hepatocellular carcinoma, Leptomeningeal carcinomatosis, Adenocarcinoma, Glioblastoma, solid tumors, melanoma (vaccination); and other diseases, such as Coronary artery disease, Amyotrophic lateral sclerosis (ALS), Rheumatoid arthritis, and Pathogenic disorders, including but not limited to, HIV, Viral Infections, Hepatitis, Non-specific bacerial infectino, tuberculosis, Herpes, Chiamyodiosis and Gastoinstinal ulcer.

Table 4: Genes Useful for Gene Therapy of the Indicated Diseases and Disorders

| GENE | DISEASE |
|---|---|
| HIF-1 alpha/VP16 | Peripheral artery disease<br>Coronary artery disease |
| PDGF | Foot ulcer<br>Venous leg ulcer |
| VEGF | Coronary artery disease<br>Peripheral artery disease |
| IL-2 | Cancer<br>Mesothelioma<br>Prostate cancer<br>Melanoma<br>Non-small cell lung cancer<br>Colon Carcinoma<br>Head and neck squamous cell cancer |
| HLA-By/beta 2-microglobulin | Melanoma |
| MDR-1 | Breast cancer |
| IL-12 | Melanoma |
| IL-112 and CD-80 | Melanoma |
| p53 | Post-hepatitus liver cancer<br>Liver cancer<br>Head and neck cancer |
| HSV-TK | Glioblastoma<br>Melanoma<br>Leukemia<br>Lymphoma<br>Pancreatic cancer<br>Liver cancer |
| IL-2 and GM-CSF | Melanoma |
| IL-2 and HLA-B7 | Renal cell cancer |
| IL-7 and IL-2 | Melanoma<br>Lymphoma<br>Renal cell cancer<br>Colon cancer |
| Cytochrome p450 | Pancreatic adenocarcinoma<br>Breast cancer |
| IL-4 | Metastatic melanoma |
| IFN-beta | glioblastoma<br>astrocytoma |
| GM-CSF | Renal cell cancer |
| IL-6 | Melanoma |
| HLA-A1 | Ovarian cancer<br>Cervical cancer |

| | |
|---|---|
| CD | breast cancer |
| Nitroreductase | Head and neck cancer<br>Liver cancer |
| ICP34.5 deleted | Glioblastoma |
| CEA | Breast cancer<br>Gastrointestinal cancer<br>Lung cancer |
| Cytidine deamininase | Colon carcinoma<br>Liver cancer |
| sFv against c-erbB-2 | Ovarian cancer<br>Breast cancer<br>Lung cancer |
| CD80 | Colorectal cancer |
| BRCA-1 | Ovarian cancer<br>Prostate cancer<br>Breast cancer |
| E1A | Breast cancer<br>Ovarian cancer over expressing c-erbB-2 |
| Mov-gamma | Ovarian cancer |
| TNF and NeoR | Melanoma |
| TNF | Melanoma |
| MART-1 | Melanoma |
| IFN-gamma | Neuroblastoma |
| Antisense to k-ras | Non-small cell lung cancer |
| PSA | Prostate cancer |
| Rb | Bladder cancer |
| MGMT | Nervous system tumors |
| HyR and HSV-TK | Prostate cancer |
| Antisense to BCR/ABL and DHFR | Chronic myelogenous leukemia |
| CC49-zeta TcR | Colorectal carcinoma |
| Tumor idiotype | Non-Hodgkin's B-cell lymphoma |
| gp100 | Melanoma |
| Her-2/neu (c-erbB-2) | Squamous cell carcinoma |
| MUC-1 | Breast cancer |
| EGF receptor | Squamous cell carcinoma |
| Tyrosinase | Melanoma |
| Cytosine deamininase and HSV-TK | Prostate cancer |
| TGF-beta and IL-2 | Non-small cell lung cancer |
| p16 | Prostate cancer |

In some instances, cancer cells upregulate specific antigens which are expressed on the cell surface. The increased expression and exposure of these "tumor specific antigens" have been targets for the development of specific binding ligands which are coupled to toxins, radiolabels, etc. and used to specifically home to

and irradicate the cancer cells. pIgR ligands can be used to target gene vectors in a similar manner to specifically treat tumors which upregulate the number of pIgR molecules on their cell surface.

## IV. VIRAL VECTORS FOR GENE DELIVERY

### A. VIRUSES

A "virus" is an obligate intracellular parasite of living but non-cellular nature, characterized by the inability to be reproduced outside of a living host cell. Inside an infected (host) cell, a virus usurps control of the cell's biosynthetic machinery and uses it to produce a multitude of progeny virus. For purposes of the present disclosure, bacterial viruses (bacteriophages) are not encompassed by the term virus. A "wildtype virus" is any virus that can be isolated from nature; alternatively, a wildtype virus is cultured in vitro but is nonetheless capable of producing progeny genomes or virions like those isolated from nature.

Viruses are classified in various manners according to different classification schemes. Under the Baltimore classification, 6 general classes of viruses are known. Class I and II viruses have DNA genomes, double-stranded (ds) in the case of Class I and single-stranded (ss) in the case of Class II. Class I viruses include but are not limited to papovaviruses, adenoviruses, herpesviruses, large bacteriophages, and poxviruses. An adenovirus is an icosahedral (20-sided) virus. There are over 40 different adenovirus varieties, some of which cause the common cold. Viruses of classes III, IV, V and VI have RNA genomes. Class III viruses have a dsRNA genome. Viruses of class IV have a positive ssRNA genome, the genome itself acting as mRNA. Class V viruses have a negative ssRNA genome that serves as a template for mRNA synthesis. Like Class IV viruses, Class VI viruses have a positive ssRNA genome but, unlike Class IV, generate a DNA intermediate not only in replication but also in mRNA synthesis.

A "retrovirus" is a Class VI virus with a single-stranded RNA genome that encodes and, upon infecting a cell, generates an enzyme known as reverse transcriptase. This enzyme catalyzes the production of dsDNA from the viral RNA template. The dsDNA form of the viral genome integrates into the host cell's chromosomes where it serves as a template for the production of ssRNA genomes,

two of which are packaged into each progeny virion. Three subclasses of retroviruses are Lentivirinae, Oncovirinae, and Spumavirinae.

The majority of viruses are recognized by the diseases they cause in plants, animals and prokaryotes, and by their infective range (i.e., their ability to infect cells from various species). Manipulation of viral production, via techniques such as DNA cloning and molecular biology, can be used to alter the infective range of a viral gene delivery vector. An "ecotropic" virus is one which can only replicate in its original host species, whereas a "xenotropic" virus is one that can be grown only on cells of a species foreign to the normal host species. Amphotropic viral delivery vectors are able to infect the cells of their host and the cells of other species. Murine amphotropic viral vectors, recombinant virions that infect mice and human cells, and murine xenotropic viruses, which are produced by but do not infect murine cells, have been derived from ecotropic viruses of wildtype mice.

**Viral Gene Delivery Vectors**

Various gene delivery systems derived from viruses have been described. Although virions containing therapeutic nucleic acids can be prepared in a variety of ways, defective viral genomes are typically used. A "defective viral genome" is a viral genome that naturally does not, or has been genetically engineered to not, serve as a template for nucleic acids and/or to not encode viral proteins required for viral replication and/or packaging. Such a genome does not produce progeny virus by itself, but may nevertheless be replicated and packaged into a virion when it is introduced into a host cell that has been co-infected with a "helper" virus, or a host cell that is a packaging cell line. A "defective virus" comprises a defective viral genome. A "satellite virus" is a naturally-occurring type of "defective virus." A non-limiting example of a satellite virus is adeno-associated virus (AAV).

A defective viral genome may, typically as a result of recombinant genetic manipulation, contain nucleic acid sequences that are or serve as a template for the production of biologically active nucleic acids, and/or encode biologically active proteins. The latter type of defective viral genome is a therapeutic defective viral genome. A "therapeutic defective virus" comprises a therapeutic defective viral genome.

A "helper virus" is a virus that will support the replication and/or packaging into a virion of a co-infecting defective virus by providing or producing the requisite proteins and/or nucleic acids that a defective virus lacks. A non-limiting example of a helper virus is adenovirus, which supports the replication and packaging of AAV.

**Virions**

A "virion" is a virus particle as it exists freely outside a cell. Virions range in diameter from about 20-about 300 nm. Structurally, a virion consists of a nucleic acid and a protein coat. Some virions are also surrounded by one or more membranes. Virions comprise viral genomic nucleic acids (in the case of wildtype viruses) or nucleic acids that are gene therapy constructs. Pseudovirions contain nucleic acids and can deliver the nucleic acids to cells, but are non-infective in the sense that they are, in the absence of helper virus or functions, incapable of replication and/or packaging. A virion-like structure that lacks viral nucleic acid is an "empty virion."

In the present disclosure, virions are categorized as enveloped or non-enveloped. Enveloped virions have an outer surface that is a membrane, i.e., a lipid bilayer. Typically, a viral membrane results from the "pinching off" or "budding off" of viral particles from an infected host cell.

Non-enveloped viruses are usually contained within a structure (a "nucleocapsid") that comprises a viral genomic nucleic acid surrounded by a protein coat. Some virions consist of a nucleocapsid surrounded by a membrane envelope; such virions are encompassed within the term "enveloped virions" as that term is used herein.

**Viral Vectors**

A "vector" is a nucleic acid molecule (typically DNA or RNA) that serves to transfer a passenger nucleic acid sequence (i.e., DNA or RNA) into a host cell. Three common types of vectors include plasmids, phages and viruses. Preferably, the vector is a virus.

## B. ENVELOPED VIRIONS

### 1. RETROVIRAL VECTORS

Retroviral genomes are RNA molecules that, during infection, produce a double-stranded (ds) DNA version of the genome. The process of producing DNA from RNA is termed reverse transcription, and the viral enzyme that catalyzes the reaction is reverse transcriptase (RT). RNase H (H, for hybrid) degrades the RNA portion of the RNA:DNA hybrid molecules that are generated by reverse transcription, after which the remaining single-stranded DNA is made double-stranded by DNA replication. The dsDNA enters the cell nucleus and, via a reaction catalyzed by viral integrase (IN), integrates into the host genome, where it serves as a template for the production of full-length viral RNA molecules via (forward) transcription. Full-length viral transcipts are often referred to as genomic RNA's. The genomic RNA's are multi-functional; they serve as mRNA molecules that have sequences that encode reverse transcriptases (pol), Gag proteins (gag, for group-specific antigen) and, after RNA splicing, envelope proteins (env). The RNA genomes further comprise signals (psi sequences) necessary for packaging the RNA into nucleocapsids. For a review, see Palu et al., "Progress With Retroviral Gene Vectors," Rev. Med. Virol. 10:185-202, 2000; see also Figure 6.

The structure of retrovirions comprises a membrane-bounded vesicle that surrounds a nucleocapsid, i.e., a proteinaceous capsid that surrounds the RNA genome and viral proteins such as RT, IN and RNase H. The nucleocapsid, which comprises Gag proteins, is further enclosed by a host cell-derived membrane. Viral transmembrane (envelope) proteins are inserted into this membrane, and project polypeptides that mediate attachment of the virions to cells. The transmembrane proteins are typically glycosylated during their production in the endoplasmic reticulum and Golgi apparatus of a host cell and are thus referred to as glycoproteins (gp).

Attachement of retrovirions to cells occurs by the interaction between viral surface (SU) proteins encoded by the retroviral gene, env, and host cell membrane proteins that act as receptors. Unless the infection spectrum of the vector virus is modified, vectors derived from viruses will deliver genes to the same cell types as the original virus.

Concurrent productive infection of cells with two types of enveloped virus can lead to the production of mixed viral particles or "pseudotypes". These naturally produced "pseudotyped" viral particles carry the nucleocapsid (including the genome) of one virus and the surface proteins of the other virus (Weiss, R. A., In: The Retroviridae, 2:1-108, ed. J. A. Levy, Plenum Press, New York (1993)).

Several commonly used retroviral vectors are derived from murine leukemia virus (MLV), a retrovirus that is able to infect many different cell types and can be produced in high titer systems. To alter the ability of viruses to target particular cell types pseudotyped vectors are produced that consist of the nucleocapsid of MLV based RV systems and the envelope of a second retrovirus or other enveloped virus having a defined targeting capacity. Pseudotyped viruses have an altered infection spectrum, since they are able to infect the same cells as the second virus that provides the envelope and/or surface proteins.

Pseudotyped RVs have been produced using packaging cell lines that produce gag and pol proteins from one virus and env proteins from a second virus. For example, nontargeted, pseudotyped RVs based upon MLV and carrying the envelope protein of the highly promiscuous vesicular stomatis virus (VSV) have been described (Yee, J. K. et. al., Proc. Natl. Acad. Sci. USA, 91:9564-9568 (1994)). These MLV/VSV pseudotyped RVs show a very wide infection spectrum and are able to infect even fish cells which suggests that, if such vectors were used for gene therapy, they would be capable of infecting many non-target cells. As other non-limiting examples, MuLV particles that have been pseudotyped with the envelope glycoproteins of HIV, or those of mouse mammary tumor virus (MMTV) have been described by, respectively, Schnierle et al., PROC. NATL. ACAD. SCI. USA 94:8640-8645, 1997; and U.S. patent No. 6,117,681 to Salmons et al.

Pseudotyped retroviral vectors based upon MoMuLV (MLV) and displaying the envelope of gibbon ape Leukemia virus (GaLV SEATO-MoMuLV hybrid virions) or the HTLV-I envelope protein (HTLV-I MoMuLV hybrid virions) have been described (Wilson, C. et. al., J. of Virology, 63(5):2374-2378 (1989)). The GaLV SEATO-MoMuLV hybrid particles are generated at titers approximately equivalent to those obtained with the MoMuLV particles, and the infection spectrum correlates

exactly with the previously reported in vitro host range of wild type GaLV SEATO (i.e., bat, mink, bovine and human cells).

**2. Ex Vivo Uses**

Retroviral vectors for gene therapy are currently used in methods for the delivery of therapeutic nucleic acids in a variety of approved protocols both in the USA and in Europe (Kotani, H., et. al., Human Gene Therapy, 5:19-28 (1994)). Most of these protocols require that the infection of target cells with the retorvirion carrying the therapeutic gene occurs in vitro, after which successfully infected cells are returned to the affected individual (Rosenberg, S. A. et. al., Human Gene Therapy, 3:75-90 (1992); (Anderson, W. F., "Human Gene Therapy", Science, 256:808-813 (1992)). Such ex vivo gene therapy protocols are suitable for medical conditions in which the target cell population can be easily isolated (e.g., lymphocytes; see Culver et al., Proc. Natl. Acad. Sci. USA 88:3155-3159, 1991; hematopoietic stem cells, see Migita et al., Proc. Natl. Acad. Sci. USA 92:1207-12079, 1995). The ex vivo infection of target cells allows the administration of large quantities of concentrated virus that can be tested for safety before use. However, not all involve target cells can be easily isolated, cultured and then reintroduced into a patient. Thus, ideally, *in vivo* gene therapy includes the ability to target RVs. Preferably, therapeutic genes carried by vectors should not be indiscriminately delivered to in all tissues and cells, but rather only to the desired target cells.

**3. Types of retroviruses used to generate viral vectors**

(a) Oncoviruses that do not contain transforming sequences, e.g. viruses of the murine (MuLV), feline (FeLV) and avian (ALV) group. It is from this group that most retroviral vectors have been derived. A RNA virus of the subfamily Oncovirinae is desirably a human T-lymphotropic virus type 1 or 2 (i.e., HTLV-1 or HTLV-2) or bovine leukemia virus (BLV), an avian leukosis-sarcoma virus, avian myeloblastosis virus (AMV), avian erythroblastosis virus (AEV), and Rous-associated virus (RAV; RAV-0 to RAV-50), a mammalian C-type virus (e.g., Moloney murine leukemia virus (MuLV), Harvey murine sarcoma virus (HaMSV), Abelson murine leukemia virus (A-MuLV), AKR-MuLV, feline leukemia virus (FeLV), simian sarcoma virus, reticuloendotheliosis virus (REV), spleen necrosis virus (SNV)), a B-

type virus (e.g., mouse mammary tumor virus (MMTV)), and a D-type virus (e.g., Mason-Pfizer monkey virus (MPMV) and "SAIDS" viruses).

(b) Onconviruses that contain oncogenic sequences derived from cellular sequences via recombination. T the v-src oncogene is, for example, found within the Rous sarcoma virus (RSV), a replication competent virus.

(c) Oncoviruses that belong to the Bovine Leukemia Virus (BLV) or Human T-Cell Lymphortopic Virus (HTLV) subgroups. The gene products of these viruses, which have complex genomes, have trans-activating and transforming properties.

(d) Spumaviruses, which include mammary tumor virus.

(e) Lentiviruses, which are a group of viruses that include immunosuppressive viruses like HIV, and viruses associated with inflammatory and degenerative diseases of animals. Lentiviruses have complex genomes, and some gene products have trans-activating functions. Because many lentiviral gene delivery vectors exist or are being pursued, the following section deals with them apart from other retroviral vectors.

**4. Lentiviruses**

Lentiviral vectors infect both dividing and non-dividing cells as they have nuclear localication signals on the capsid molecule which allow for their active import into the nucleus without requring cell division and breakdown of the nuclear envelope. They allow for persistent transgene expression; however, the development of their technology has logged behind that for retroviral vectors due to safety concerns. These vectors also tend to be less immunogenic, and have been used for i.v., i.p., i.m. as well as introtumoral injections. For a review, see Lever, "HIV and Other Lentivirus-Based Vectors," Gene Ther. 3:470-471, 1996.

A RNA virus of the subfamily Lentivirus is desirably a human immunodeficiency virus type 1 or 2 (i.e., HIV-1 or HIV-2, wherein HIV-1 was formerly called lymphadenopathy associated virus 3 (HTLV-III) and acquired immune deficiency syndrome (AIDS)-related virus (ARV)), or another virus related to HIV-1 or HIV-2 that has been identified and associated with AIDS or AIDS-like disease. The acronym "HIV" or terms "AIDS virus" or "human immunodeficiency virus" are used herein to refer to these HIV viruses, and HIV-related and associated viruses,

generically. Moreover, a RNA virus of the subfamily Lentivirus preferably is a Visna/maedi virus (e.g., such as infect sheep), a feline immunodeficiency virus (FIV), bovine lentivirus, simian immunodeficiency virus (SIV), an equine infectious anemia virus (EIAV), and a caprine arthritis-encephalitis virus (CAEV). HIV-derived vectors for anti-HIV gene therapy have been described (Poeschla et al., PROC. NATL. ACAD. SCI. USA 93:11395-11399, 1996).

**5. Spleen Necrosis Virus (SNV)**

Spleen necrosis virus (SNV) is an amphotropic retrovirus originally isolated from a duck. Although of avian origin, it also replicates on some mammalian cells (Koo et al., "Spleen Necrosis Virus, An Avian Retrovirus, Can Infect Primate Cells," J Virol 1991, 65:4769-76). SNV-derived retroviral vectors work with relatively high efficiency and have potential as gene delivery vectors, particularly as it is capable of infecting and transforming non-dividing cells. See, e.g., Parveen et al., "Spleen Necrosis Virus-Derived C-Type Retroviral Vectors For Gene Transfer To Quiescent Cells," Nat. Biotechnol 2000, 18:623-9; Jiang et al., "A Genetically Engineered Spleen Necrosis Virus-Derived Retroviral Vector That Displays The Hiv Type 1 Glycoprotein 120 Envelope Peptide," Hum. Gene Ther. 1999,10:2627-36; Olson et al., "Improved Self-Inactivating Retroviral Vectors Derived From Spleen Necrosis Virus," J. Virol 1994, 68:7060-6; Kewalramani et al., "Spleen Necrosis Virus, An Avian Immunosuppressive Retrovirus, Shares A Receptor With The Type D Simian Retroviruses," J. Virol 1992, 66:3026-31; and Koo et al., "A Spleen Necrosis Virus-Based Retroviral Vector Which Expresses Two Genes From A Dicistronic mRNA," Virology 1992, 186:669-75.

**6. Disabled Viral Vectors and Packaging Cell Lines**

Retroviral and other viral vectors that are capable of accommodating relatively long stretches of therapeutic nucleic acids, including those that are disabled (i.e., incapable of autoreplication, self-packaging, etc.) have been developed (Figure 11). Such vectors typically have at least some non-essential viral sequences deleted so as to allow the incorporation of larger stretches of therapeutic nucleic acids in the viral genome. Disabled vectors have deletions of genes required for replication and/or packaging. Production of disabled viruses requires two components, a packaging cell line and a disabled vector.

A packaging cell line (Figures 9 and 10) contains all the structural genes for virions but is deleted for packaging (psi) sequences; thus, by itself, the cell line thus releases virions lacking a viral RNA genome. Transfection of these packaging cells with a disabled retrovirus vector, the genome of which contains packaging sequences, results in incorporation of the vector RNA into virus particles. These virions may infect cells, and integrate a DNA copy of the vector genome into the target cell's genome, but are incapable of further replication.

Transfection of a vector into the first generation packaging cell lines described above can, through recombination, result in the production of replication-competent retrovirus (RCR). Wildtype viruses can even be found in harvests made after transient transfections of this type of packaging cell line and, efforts have been directed at improving their safety. Second generation packaging cell lines such as PA317 are improved in the sense that, in addition to deletion of the packaging sequence, the 3'LTR is also deleted so that two recombination events are necessary to generate a wild type virus; nevertheless, RCR can be generated in these cells.

The most advanced and safe packaging cell lines have "split genomes" where the gag-pol gene and env gene are present in two or more physically distinct expression constructs, which significantly reduces the frequency of recombination between vector and packaging sequences. Typically, to construct such cell lines, a gag-pol expression construct is first introduced into a cell line of interest (e.g., HT1083 or 293 cells), and stable transformants are isolated and sublconed via dilution. An expression construct comprising an env gene is then introduced into isolated subclones, and transformants are assayed for their ability to stably produce env proteins. Stable double tranformants are assayed for the titer of empty virions that are produced thereform, and subclones that produce high levels of virions are identified and isolated. Techniques for producing and using retroviral packaging cell lines are described by Cepko, Section III, Transduction of Genes Using Retroviral Systems, of Chapter 9 In: Short Protocols in Molecular Biology (2nd ed.), Ausubel et al., Eds., John Wiley & Sons, New York, NY, pages 9-30 to 9-48, 1992.

A variety of retroviral packaging cell lines have been described; see, for example, U.S. Patent No. 5,985,655; Meyers et al., Development And Testing Of A Packaging Cell Line For Avian Retroviral Vectors, Arch. Virol 1991, 119:257-64;

Ban et al., Bovine Leukemia Virus Packaging Cell Line For Retrovirus-Mediated Gene Transfer, J. Gen. Virol 1989, 70:1987-93; and Watanabe et al., Construction Of A Helper Cell Line For Avian Reticuloendotheliosis Virus Cloning Vectors, Mol. Cell Biol. 1983, 3:2241-9. For replication defective viruses the specificity of the envelope of glycoproteins of the vector virus can be altered by changing the sequences expressed within the packaging cell line. For example, the envelope glycoprotein of vesicular stomatitis virus can be incorporated into ecotropic MuLV and will widen the infective range of the virions.

## C. HERPES VIRUS VECTORS

Herpes simplex (HSV) is a double-stranded DNA virus. Primary infection normally results in productive infection in epithelium which probably involves millions of cells. HSV has a tropism for neuronal tissue, and is thus particularly useful for treatments of the central nervous system (CNS). The restriction of HSV to the epithelium and sensory nerves is not due to receptor specificity as HSV is capable of binding to and entering a wide range of cell types.

### 1. Disabled HSV vectors and packaging cell lines

Approximately half of the 70-odd genes of HSV are dispensable for growth in vitro. Deletion or disruption of these genes has, in many cases, been demonstrated to result in substantial attenuation in the mouse. However, careful review of the evidence supporting the attenuation will be needed if particular insertion sites are used. Insertion into, or disruption of, the following genes have been shown to cause substantial attenuation: Thymidine kinase (gene UL23); Ribonucleotide reductase (gene UL39, UL40); Gamma 34.5 (gene RL1); IE-110 (ICPO) (gene RL2); IE-4 (ICP22) (gene US 1); Protein Kinase (gene US-3); Glycoprotein I (gene US-7); and Glycoprotein E (gene US-8).

Replication defective HSV vectors, e.g., deletion mutants lacking essential replication genes have been constructed. A non-limiting example of such vectors is the gH-vector DISC HSV-1 which, together with appropriate helper cell lines providing the relevant functions in trans, provides a non-replicating HSV virion. Mutants of this type have the potential to act as replication defective vectors and, provided the foreign gene is inserted at the site of disablement, can be considered to

provide a high margin of safety. Packaging cell lines for HSV have been described; see, e.g., Geller et al., PROC. NATL. ACAD. SCI. USA 87:8950-8954, 1990).

**2. Amplicons**

"Amplicons" are plasmids carrying an HSV origin of replication and packaging sites, and can be used as packagable vectors to deliver foreign genes. Since they carry no viral genes they are not cytotoxic and can be used as gene delivery vehicles. By definition they are helper virus dependent and stocks usually contain at least 50% helper virus.

## D. ALPHAVIRUSES

Alphaviruses comprise several anthopod-borne viruses in the family Togaviridae. Alphaviruses have a single stranded, positive sense RNA genome which is enclosed in a capsid protein. Two glycoproteins, E1 and E2, are incorporated into the membrane that envelopes the capsid. The virus forms two polyprotein products, one translated from the viral 5' end sequence, the other from transcription of a subgenomic mRNA corresponding to the 3' third of the genome, from where the encoded proteins are processed in infected cells to their mature forms and are incorporated into the infectious virus particles.

Alphaviruses are enzootic, naturally infecting and replicating in mosquitoes as well as other animal species including birds. Humans infected with Hazard Group 2 alphaviruses (e.g., Semliki Forest [SFV], Sindbis [SIN] or Ross River virus) may develop mild symptoms, but recovery is usually uncomplicated and complete. On the other hand, Venezuelan, Eastern and Western equine encephalitis virus are Hazard group 3 viruses and may produce epidemics of encephalitis in horses or even humans with high mortality rates. SFC and the similar SIN are likely to be used as vectors for genetic modification.

Alphaviruses (positive strand RNA viruses) such as Sinbus, Semliki Forrest and VEE viruses are suicide viruses in that they produce a lot of recombinant protein once they infect a cell, however, the production is transient as they kill they cell they infect and hence the protein factory. Some alphaviruses, such as VEE, have an endogenous tropism for dendritic cells, a feature that results in a more rapid immune response. They thus have the potential utility as, inter alia, vaccines as they produce

a lot of an immunogenic antigen for a transient period of time, which one would want to illicit an immune response and build up tolerance in an individual.

The Semliki forest virus (SFV) is an enveloped virus of the alphavirus group of Togaviridae. The virus was isolated from mosquitoes in the Semliki Forest in Uganda and is not known to cause any illness. The synthesis and export of the virus's three spike glycoproteins has been studied as a model for the synthesis and export of plasma membrane proteins.

Disabled or attenuated alphavirus vector systems, inlcuding disabled derivatives of SFV, have been produced which ensure that the recombinant virus undergoes only one cycle of infection. In vitro transcription of the plasmid clones generates a packaging competent replicon RNA which also encodes the foreign gene(s) and a packaging incompetent DI helper RNA that encodes the virus structural proteins. These RNAs are co-transfected into permissive cells to generate recombinant, infectious virus capable of only a single replicative cycle. Although the replicon RNA persists in infected cells, no virus can be produced and the extent of the infection is limited to those cells initially exposed to virus. However, replication competent virus (RCV) may be produced by recombination in the packaging cell lines (thought to be due to replicate strand switching between replicon and helper RNAs). A further modification to this system involves the use of strains with a mutation in the p62 spike protein and packaged viruses require in virtro treatment with chymotrypsin before they are able to infect susceptible cell lines (Berglund et al., Biotechnology 11:916-920, 1993). Stable alphavirus packaging cell lines, including but not limited to those for Semliki Forest and Sindbis gene delivery vectors, have been described (Polo et al., Proc. Natl. Acad. Sci. USA 96:4598-4603, 1999).

In a further system (which is not disabled), a full length cDNA copy of the viral RNA is modified to contain a second internal subgenomic RNA promoter positioned downstream of the internal RNA promoter which expresses the structural proteins. Heterologous gene sequences are inserted immediately downstream of the second promoter. This cDNA plasmid template is transcribed in vitro and when the resultant RNA is transfected into susceptible cells a fully infectious alphavirus that expresses the heterologous gene(s) is produced. Recombinant infectious clones using

such vectors are generally less stable, losing their inserts on repeated passage mainly due to the lack of editing function in the RNA dependent RNA polymerase.

For reviews of alphaviral gene delivery vectors, see Polo et al., "Stable Alphavirus Packaging Cell Lines for Sindbi Virus and Semiliki Forest Virus-Derived Vectors," Proc. Natl. Acad. Sci. USA 96:4598-4603, 1999; and K. Lundtrom, "Alphaviruses as Tools in Neurobiology and Gene Therapy," J. Recept. Signal Transduct Res. 19:673-86, 1999. For reviews and examples of Sendai viral delivery vectors, see Ramani et al., "Site-Specific Gene Delivery *In Vivo* Through Engineered Sendai Viral Envelopes," Proc. Natl. Acad. Sci., USA, Vol. 95, pp. 11886-11890, 1998; Nakanishi et al., "Gene Delivery Systems Using the Sendai Virus," Mol. Membr. Biol., Vol. 16:123-127, 1999; and Ramani et al., Proc. Natl. Acad. Sci. USA 95:11886-11890, 1998.

## E. NON-ENVELOPED VIRIONS (CAPSIDS)

### 1. ADENOVIRAL VECTORS

Human adenovirus is a double-stranded DNA virus. Adenoviral virions are protein capsids in the shape of icosahedrons that are about 65 to 80 nanometers (nm) in diameter. The capsid is composed of 252 morphological units (capsomeres) that are hexameric (240 hexons) or pentameric (12 pentons). Fiber proteins project from the penton bases via noncovalent interactions (Boudin et al., Virology 92:125-138, 1979). For a review, see Benihoud et al., Adenovirus Vectors for Gene Delivery, Current Op. Biotech. 10:440-447, 1999)

Interactions between capsid proteins (particularly fiber proteins and penton base proteins) and distinct cellular receptors mediate the attachment and entry of virions into cells (Wickham et al., Cell 73:309-319, 1993). In order to attach a virus to a cell, wildtype fiber proteins bind to the cellular receptor known as the coxsackievirus and adenovirus receptor (CAR). After attachment to the cell via fiber proteins, penton base protein binding to proteins known as integrins mediates the internalization of virions into clathrin-coated endocytic vesicles.

Integrins are proteins that mediate cellular adhesion to extracellular matrix (ECM) proteins (e.g., fibronectin, vitronectin, laminin, collagen, etc.) that often specifically bind short amino acid sequences such as the tripeptide RGD (Arg Gly

Asp). A similar interaction with integrins, especially the alphavbeta3/5 subtype of integrins, seems to be involved in adenoviral entry, as exogenously added RGD peptides block penton base binding, and adenoviruses that have point mutations in the RGD sequence of the penton base protein have a restricted ability to infect cells (Bai et al., J. Virol., 67:5198-5205, 1993). Different viral serotypes may result from the use of different cellular receptors and integrins. In general, alphavbeta3/5 integrins are down-regulated in epithelial cells, and are thus displayed in lower amounts, if at all, on epithelial cells. Integrins are thus are not optimal targets for adenoviral-mediated gene delivery. In contrast, pIgR, pIgR stalk and pIgR secretory component (SC)proteins are abundant on epithelial cells and are relatively underexpressed in other types of cells. It is thus believed by applicants that gene delivery vectors targeted to pIgR, pIgR stalk, and pIgR SC molecules will be most effective for delivering nucleic acids to epithelial cells.

Adenoviruses have efficient mechanisms for transport from endocytotic vesicles to the cytoplasm. Viral particles are ultimately transported to the nuclear pore complex of the cell, by which the viral genome enters the nucleus (Figure 7). The ability of adenovirus to efficiently enter cells can allow for efficient adenoviral-mediated targeted transfer of genes to the cells or tissues of individuals suffering from a particular disease or disorder.

Inside the cell, the lytic cycle of adenovirus is divided into the early and late phases. "Early genes," including the E1a and E1b transforming genes are first expressed, leading to replication of the ~36 kb viral genome as a linear episome. Subsequently, expression of the "late" genes encoding capsid proteins occurs. Vectors based on disabled (defective) adenovirus typically have a deletion removing most of the E1 region, thus preventing expression of the E1a and E1b genes.

Between ~100 and $10^5$ viral particles may be produced per cell in a lytic cycle that lasts from about 24 to about 48 hours. Although adenoviruses have no mechanism for cytolysis, and many viral particles remain associated with cells in culture, sufficient particles are released or otherwise gain access to infect neighboring cells. After several rounds of replication in confluent cell cultures, visible plaques of infected cells, in which the cells show typical cytopathic effects (rounding up the cytoplasm and clumping into grape-like clusters) are produced.

Adenoviruses have the potential to infect a wide variety of cell types, although in terms of their natural pathogenicity, they are generally grouped according to their association with respiratory or enteric diseases. A potential limitation to the use of adenovirus in gene therapy is that all cells (not just the cells in need of therapeutic treatment) that display both CAR and the appropriate integrin will internalize adenoviral virions gene(s) being administered; moreover, cells that lack either one or both of these binding targets are not generally amenable to adenoviral-mediated gene delivery. See, for example, Silver et al., Virology, 165:377-387, 1988; Horvath et al., J. Virol. 62:341-345, 1988; Cotten et al., Proc. Natl. Acad. Sci. (USA) 87:4033-4037, 1990; Huang et al., J. Virol. 69:2257-2263, 1995; and Wattel et al., Leukemia 10:171-174, 1996. In order to modify the binding characteristics of adenovirions, the fiber protein has been modified to incorporate amino acid sequences that interact directly or indirectly with cell receptors. Such amino acid sequences include, respectively, those that act as a ligand to a cell surface receptor, or those that allow the adenovirion to bind to one variable region of a bispecific antibody, where the other variable region present in the antibody binds a cellular receptor (see, e.g., PCT International Patent Application WO 95/26412). In both cases, the wildtype fiber/cell surface receptor interactions do not occur, and adenovirions are directed to a cell surface receptor of choice by means of the modified fiber protein. If the targeted tissue is the lung, adenoviral vectors may be preferred based on their normal tropism for the respiratory epithelium.

Compositions and methods for producing adenovirions with altered specificity are, by way of non-limiting example, described in U.S. Patent No. 5,559,099, to Wickham et al., entitled "Penton Based Protein And Methods Of Using Same" issued on September 24, 1996; U.S. Patent No. 5,770,442, to Wickham et al., entitled "Chimeric Adenoviral Fiber Protein And Methods Of Using Same" issued on June 23, 1998; U.S. Patent No. 6,057,155, to Wickham et al., entitled "Targeting Adenovirus With Use Of Constrained Peptide Motifs" issued on May 2, 2000; U.S. Patent No. 6,127,525, to Crystal et al., entitled "Chimeric Adenoviral Coat Protein and Methods of Using Same, issued on October 30, 2000; Fender et al., Adenovirus Dodecahedron, A New Vector For Human Gene Transfer, Nat. Biotechnol, Vol. 15, No. 1; pp. 52-56, 1997; Dmitriev et al., An Adenovirus Vector With Genetically

Modified Fibers Demonstrates Expanded and Adenovirus Utilization of a Coxsackievirus and Adenovirus Receptor-Independent Cell Entry Mechanism, J. Virol, Vol. 72, No. 12, pp. 9706-9713, 1998; Michael et al., Addition of A Short Peptide Ligand to the Adenovirus Fiber Protein, Gene Ther. Vol. 2, No. 9, pp. 660-668, 1995.

Genetically modified viruses are typically produced by manipulation of partial viral genomes in bacterial plasmids. Overlapping plasmids are co-transfected into cells (293 cells are often used) wherein complete genomes may be generated by homologous recombination. Alternatively, linearized plasmid DNA is ligated in vitro ligation in order to regenerate the full length viral genome prior to transfection.

Advantages that accompany the use of adenoviruses as vectors for gene therapy include (1) recombination is rarely observed with adenoviruses; (2) there is no ostensible correlation of any human malignancy with adenoviral infections despite common human infection with adenoviruses; (3) the adenoviral genome (which is comprised of linear, double-stranded DNA) can be manipulated to carry up to about 7.5 kb of exogenous DNA, and longer DNA sequences can potentially be carried into a cell, for instance, attached to the adenoviral capsid (Curiel et al., Human Gene Therapy, 3, 147-154 (1992)); (4) an adenovirus is unlikely to interfere with normal cell function since the vector commands expression of its encoded sequences in an epichromosomal fashion; and (5) live adenovirus has been safely used as a human vaccine for many years.

Adenovirus and adenoviral (DNA) vectors establish themselves episomally within the nucleus (they do not integrate into the genome) and can carry large sizes of DNA. They too infect dividing and non-dividing cells and usually with higher efficiency than retroviral vectors. However, certain strains are involved in generating certain forms of the common cold, and therefore many people with develop an immune reaction and sometimes an anaphylactic reaction (and possibly death as this is the form of vector that actually killed the young man from Tuscon.) Because the virus doesn't integrate, expression is often considered transient in some cases, and repeat dosing is a problem due to the potential immunologic problems.

## 2. ADENO-ASSOCIATED VIRUS (AAV)

Adeno-associated virus (AAV) is a satellite virus, i.e., a defective parvovirus that requires co-infection with adenovirus for completion of its lytic cycle. The AAV genome is a linear single stranded (ss) DNA molecule of 4680 nucleotides that encodes Rep (replication) and Cap (capsid) proteins. Flanking the AAV coding regions are two 145 nucleotide inverted termini (ITR) repeat sequences that contain palindromic sequences that can form hairpin structures that function as primers during initiation of DNA replication. In addition to their role in DNA replication, the ITR sequences are necessary for viral integration, rescue from the host genome and encapsidation of viral nucleic acid into mature virions (Muzyczka, N., Curr. Top. Micro. Immunol. 158:97-129, 1992).

AAV capsids have icosahedral symmetry and are from about 20 to about 24 nm in diameter. The capsids are composed of three proteins (VP1, VP2, and VP3, which are approximately 87, 73 and 61 Kd, respectively) (Muzyczka, N., Curr. Top. Micro. Immunol. 158:97-129, 1992). VP3 represents 90% of the total virion protein; VP2 and VP1 account for approximately 5% each. All of the capsid proteins are N-acetylated.

AAV can assume two pathways upon infection of a host cell. In the presence of helper virus, AAV will enter the lytic pathway where the viral genome is transcribed, replicated, and encapsidated into newly formed viral particles. In the absence of helper virus function, the AAV genome becomes integrated as a provirus into a specific region of the host cell genome. Characterization of the proviral integration site and analysis of flanking cellular sequences indicates specific targeting of AAV viral DNA into the long arm of human chromosome 19 (Kotin, R. M., et al., Proc. Natl. Acad. Sci. USA 87:2211-2215, 1990; Samulski, R. J., et al., EMBO J. 10:3941-3950, 1991). The site specific integration of AAV reduces the likelihood of insertional mutagenesis resulting from random integration of viral vector DNA into the coding region of a host gene. The AAV ITR sequences appear to be devoid of transcriptional regulatory elements, which reduces the risk of insertional activation of protooncogenes.

When an intact AAV genome is cloned into a prokaryotic vector and is transfected into cells, in the presence of helper virus, AAV is rescued from the

plasmid vector and enters the lytic pathway leading to production of mature virions. In the absence of helper virus, the recombinant AAV vector will integrate into the host cell genome and remain as a provirus until the cell subsequently becomes infected with a helper virus.

AAV vectors are among a small number of recombinant virus vector systems that have been shown to have utility as both in vitro and in vivo gene transfer vectors (reviewed in Carter, 1992, Curr. Opinion Biotech. 3, 533-539 (1992); Muzcyzka, Curr. Top. Microbiol. Immunol. 158, 97-129). AAV vectors can infect dividing as well as non-dividing cells and are capable of stable DNA integration and expression in a variety of cells including cystic fibrosis (CF) bronchial and nasal epithelial cells (Flotte et al., Am. J. Respir. Cell Mol. Biol. 7, 349-356 (1992a)); Egan et al., Nature, 358, 581-584 (1992); Flotte et al., J. Biol. Chem. 268, 3781-3790 (1993a); Flotte et al., Proc. Natl. Aced. Sci. U.S.A. 90, 1613-1617 (1993b), human bone marrow-derived erythroleukemia cells (Walsh et el., Proc. Natl. Acad. Sci. U.S.A. 89, 7257-7261 (1992)), and several others. See also Flotte et al., Proc. Natl. Acad. Sci. U.S.A., 90, 10613-10617 (1993).

AAV-derived vectors include but are not limited to those described in U.S. Patent Nos. 6,080,569; 6,156,303; 6,207,453; 6,204,059; 6,200,560; 6,180,613; 6,165,781; 6,156,303; 6,027,931; 6,004,797; 5,989,540; 5,952,221; 5,871,982; 5,863,541; 5,843,742; and 5,786,211; Smith-Arica et al., Curr Cardiol Rep. 3:43-49, 2000; Yan et al., Proc. Natl. Acad. Sci. USA, Vol. 97, No. 12, pp. 6716-6721, 2000; Rutledge et al., J. Virol. 72:309-319, 1998; and Zhou et al., Journal of Virology 72:3241-3247, 1998.

### 3. POXVIRUSES

Vaccinia virus recombinants are useful tools for the molecular biologist and immunologist. High levels of expression can be achieved facilitating biochemical, biological and immunological characterization of foreign genes. Several strains of vaccina material as part of the smallpox eradication campaign.

The complex and large genome of vaccinia (over 175Kb depending on the strain) contains an estimated 150-200 genes many of which are necessary to enable the virus to replicate in the cytoplasm of infected cells. Infectious progeny virus can

be detected approximately 6 hours after infection and continue for about 48 hours. With the commonly used strains, the progeny virus are released by eventual cell lysis.

Avipoxviruses are restricted to growth in avian species. Fowlpox, pigeonpox and canarypox have been used as vectors for foreign genes generally with the intention to use them as vaccines. Attenuated derivatives of fowlpox virus FPV (TROVAC) and canarypox virus (ALVAC) have been demonstrated to be non-virulent in a variety of immuno-suppressed animals and human volunteers. Other poxviruses have been used as vectors e.g., suipox and sheeppox/goatpox.

Within poxviruses, host range varies in nature and extent. Vaccinia host range includes humans and animal species such as cattle, cats, rodents, rabbits and pigs, although the virus does not appear to occur naturally in humans and has no animal reservoir. At least three host range genes can be identified in poxviruses; vaccina contains C7L and K1L, the insertion of a third, CHO hr gene, allows the virus to grow on rabbit kidney cells. None of the host range genes are receptor attachment proteins. Many poxvirus genes are dispensable for growth in vitro. Deletion or disruption of these genes has, in many cases, been demonstrated to result substantial attenuation in the mouse.

### 4. BACULOVIRUSES

Baculoviruses are pathogens of a range of insects and may, in certain circumstances, pose a potential threat to such species in the natural environment. In particular, the use of baculoviruses and susceptible host organisms must be given particular attention to ensure release to the environment does not occur.

The most commonly used Baculovirus vector utilizes the highly expressed and regulated Autographa californica nuclear polyhedrosis virus (AcNPV) polyhedron promoter modified for the insertion of foreign genes. One of the major advantages of this invertebrate virus vector is the very abundant expression of recombinant proteins in cell cultures such as Sf9 from Spodoptera frugiperda.

Although wildtype baculovirus is pathogenic for certain Lepidotera, expression systems are based on deletion of the polyhedrin gene. Baculoviruses can express foreign genes under the control of mammalian specific promoters in human or rat hepatocytes (Hofman et al., Proc. Natl. Acad. Sci. USA 92:10099-10103, 1995;

Boyce and BucHer, Proc. Natl. Acad. Sci. USA 93:2348-2352, 1996; Condreay et al., Proc. Natl. Acad. Sci. USA 96:127-132, 1999; U.S. Patent 5,871,986).

### 5. OTHER NON-ENVELOPED VIRUSES

Other non-enveloped viruses that have been used to create gene delivery vehicles include but are not limited to Papillomavirus (Touze et al., NAR 26:1317-1323, 1998). Methods and compostions for pseudotyping viral capsids are discussed in, for example, U.S. Patent 6,190,887.

## V. LIPOSOMES

Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layer(s) made up of lipids arranged in a bilayer configuration (see, generally, Chonn et al., Current Op. Biotech., 1995, 6, 698). Liposomes may be used as cellular delivery vehicles for bioactive agents in vitro and in vivo (Mannino et al., Biotechniques, 1988, 6, 682; Blume et al., Biochem. et Diophys. Acta, 1990, 1029, 91; Lappalainen et al., Antiviral Res., 1994, 23, 119. For example, it has been shown that large unilamellar vesicles (LUV), which range in size from about 0.2 to about 0.4 microns, can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior of liposomes and delivered to brain cells in a biologically active form (Fraley et al., Trends Biochem. Sci., 1981, 6, 77). Liposome-based gene therapy is reviewed by Tseng et al., Pharm. Sci. Tech. Today 1:206-213, 1998; and Ropert, Braz. J. Biol. Res. 32:63-169, 1999. U.S. Patent 5,834,441 is stated to describe liposomes for the delivery of AAV-derived nucleic acids.

Liposomes may be unilamellar (single layer) or multilamellar (multilayer, often compared to an onion skin) and they may be loaded with drugs, peptides, proteins, nucleic acids, carbohydrates, plasmids, vitamins, cosmetics, and the like (Bakker-Woudenberg et al., Liposomes as carriers of antimicrobial agents or immunomodulatory agents in the treatment of infections, Eur J Clin Microbiol Infect Dis 1993;12 Suppl 1:S61-67; Gregoriadis et al., Liposomes in drug delivery. Clinical, diagnostic and ophthalmic potential, Drugs 1993 45:15-28). Examples of techniques for encapsulating molecules into liposomes are described by Mayer et al.,

Techniques for encapsulating bioactive agents into liposomes, Chem Phys Lipids 40:333-345, 1986.

Liposomes make it possible to encapsulate water soluble and water insoluble substances and avoid the use of other formulations that depend on emulsification and/or surfactants. Liposomes enable the ability to control delivery characteristics of substances with the use of biodegradable and nontoxic materials that comprise the liposome formulation. While substances are contained in the liposome, they are resistant to enzymes and oxidants that exist in the vicinity of the liposome. Liposomes may be injected into a patient; intravenous or subcutaneous injection may be used. In addition, liposomes can be administered to the gastrointestinal tract or the respiratory tract. Liposomes may be encapsulated.

Liposomes are formed from vesicle-forming lipids which generally include one or more neutral or negatively charged phospholipids, typically one or more neutral phospholipids, usually in combination with one or more sterols, particularly cholesterol. Non-limiting examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, sphingolipids, phosphatidylethanolamine, cerebrosides and gangliosides.

Often, the major lipid component of liposomes is a phosphatidylcholine (PC) or PC derivative. PC derivatives with a variety of acyl chain groups of varying chain length and degree of saturation are commercially available or may be synthesized by known techniques. For purposes of filter sterilization, less-saturated PCs are generally more easily sized, particularly when the liposomes must be sized below about 0.3 microns. PCs containing saturated fatty acids with carbon chain lengths in the range of about 14 to about 22 carbon atoms are commonly used particularly diacyl phosphatidylglycerols. Illustrative phospholipids include, for example, dipalmitoylphosphatidylcholine, phosphatidylcholine and distearoylphosphatidylcholine. Phosphatidylcholines with mono- and di-unsaturated fatty acids and mixtures of saturated and unsaturated fatty acids may also be used. Other suitable phospholipids include those with head groups other than choline, such as, for example, ethanolamine, serine, glycerol and inositol. Other suitable lipids include phosphonolipids in which the fatty acids are linked to glycerol via ether

linkages rather than ester linkages. In some embodiments, liposomes include a sterol, e.g., cholesterol, at molar ratios of from about 0.1 to about 1.0 (sterol: phospholipid).

**Streically Stabilized Liposomes**

The term "sterically stabilized liposome" refers to a liposome comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome comprises one or more glycolipids, such as monosialoganglioside $G_{M1}$, or is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (Allen et al., FEBS Letts., 1987, 223, 42; Wu et al., Cancer Res., 1993, 53, 3765; Papahadjopoulos et al., Ann. N.Y. Acad. Sci., 1987, 507, 64; Gabizon et al., Proc. Natl. Acad. Sci. USA, 1988, 85, 6949; U.S. Patent No. 4,837,028 and published PCT application WO 88/04924, both to Allen et al. U.S. Pat. No. 5,543,152 to Webb et al.; and published PCT application WO 97/13499 to Lim et al.

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (Bull. Chem. Soc. Jpn., 1980, 53, 2778) describe liposomes comprising a nonionic detergent. Liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate or other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG have significant increases in blood circulation half-lives. (Blume et al. Biochimica et Biophysica Acta, 1990, 1029, 91; Klibanov et al., FEBS Letts., 1990, 268, 235). Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. 0,445,131 BE and WO 90/04384 to Fisher. Liposome compositions containing about 1 to about 20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle et al.

(U.S. Pat. Nos. 5,013,556 and 5,356,633) and Martin et al. (U.S. Pat. No. 5,213,804 and European Patent No. EP 0,496,813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Pat. No. 5,225,212 (both to Martin et al.) and in WO 94/20073 (Zalipsky et al.) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi et al.). U.S. Pat. Nos. 5,540,935 (Miyazaki et al.) and 5,556,948 (Tagawa et al.) describe PEG-containing liposomes that can be further derivatized via functional surface moieties.

**Targeting of Liposomes**

Liposomes can be either passively or actively targeted. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticuloendothelial system in organs that contain sinusoidal capillaries. Active targeting, by contrast, involves modification of the liposome by coupling thereto a specific ligand such as a viral protein coat (Morishita et al., Proc. Natl. Acad. Sci. USA, 1993, 90, 8474), monoclonal antibody (or a suitable binding portion thereof), sugar, glycolipid or protein (or a suitable oligopeptide fragment thereof), or by changing the composition and/or size of the liposome in order to achieve distribution to organs and cell types other than the naturally occurring sites of localization.

Targeting of liposomes may be achieved in a variety of ways. Various linking groups are used to join lipid chains of the liposome to a targeting element. The targeting element binds a specific cell surface molecule found predominantly on cells to which delivery of the compounds of the invention is desired. Targeting elements include, by way of non-limiting for example, a hormone, growth factor or a suitable oligopeptide fragment thereof which is bound by a specific cellular receptor predominantly displayed on by cells to which delivery is desired, or a polyclonal or monoclonal antibody, or a suitable fragment thereof (e.g., Fab; scFv) that specifically binds an antigenic epitope found predominantly on targeted cells.

The targeting of liposomes may be controlled by coating the outside surface of the liposome with targeting agents such as an antibody, F(ab')2 or Fab fragment of an antibody, cytokines, enzymes, domains and portions of proteins, peptides, polypeptides, carbohydrates, nucleic acids, oligonucleotides, etc. Such coating substances may be present in various amounts on the surface of the liposomes. In the

present invention, fusion proteins that project a pIgR ligand from a bi-layer lipid membrane are used to target liposomes.

Targeting of liposomes to different cell types can also be modulated by manipulating the type and ratio of lipids present therein. See, for example, Duzgune et al., Mechanisms and kinetics of liposome-cell interactions, Adv Drug Deliv Rev 1999 40:3-18; Schreier et al., Targeting of liposomes to cells expressing CD4 using glycosylphosphatidylinositol-anchored gp120. Influence of liposome composition on intracellular trafficking. J Biol Chem 1994 269:9090-9098; and Shi et al., Noninvasive gene targeting to the brain, Proc. Natl. Acad. Sci. USA 97:7567-7572, 2000; Shimizu et al., Formulation of liposomes with a soybean-derived sterylglucoside mixture and cholesterol for liver targeting. Biol Pharm Bull 1997 20:881-886.

**Insertion of Transmembrane Proteins into Liposomes**

Transmembrane proteins are inserted into the lipid bilayer of liposomes in a variety of ways. Purified proteins can be reconstituted into liposomes via in vitro reconstitution using biochemical methods (Slepushkin et al., Biochem Biophys Res Commun 1996 227:827-33; Brenner et al., Methods Enzymol 2000;322:243-252; Xu et al., J Membr Biol 1999 170:89-102; Genchi et al., Plant Physiol 1999 120:841-848; Lagutina et al., Biochemistry (Mosc) 1998 63:1328-1334; Orellana et al., Mimicking rubella virus particles by using recombinant envelope glycoproteins and liposomes, J Biotechnol 1999 75:209-219).

Some membrane proteins apparently insert into membranes spontaneously, i.e., on their own accord (Mel et al., Biochemistry 1993 32:2082-2089; Antonsson et al., Biochem J 2000 345:271-278). In some instances, spontaneous transmemebrane insertion of membrane proteins into liposomes may be facilitated by lecthins, particularly short-chain lecthins (Dencher, Biochemistry 1986 25:1195-1200).

Membrane-spanning and/or membrane-inserting synthetic amino acid sequences can be prepared by in silico design and in vitro experimentation. See, e.g., Wimley et al., Biochemistry 2000 39:4432-4442; Chung et al., Biochemistry 1996;35:11343-11354; Bormann et al., J Biol Chem 1989 264:4033-4037; Percot et al., Design and characterization of anchoring amphiphilic peptides and their interactions with lipid vesicles, Biopolymers 1999 50:647-655; and Chakrabarti et al.,

Influence of charge, charge distribution, and hydrophobicity on the transport of short model peptides into liposomes in response to transmembrane pH gradients, Biochemistry 1994 33:8479-8485.

Transmembrane and membrane-directing amino acid sequences from such proteins are included in fusion proteins that further comprise a targeting element. Such fusion proteins can be introduced into the membranes of liposomes or of enveloped virions.

As used herein, the term "transmembrane" refers to amino acid sequence that traverse both of the lipid layers of a membrane, as well as "anchored" proteins which comprise a lipophilic moiety that may be incorporated into the outer lipid layer of a membrane. Membrane anchoring structures direct the fusion protein to a liposomal or viral membrane, where it is preferably anchored in the outer lipid layer, projecting into the environment surrounding such membrane-bounded vesicles. For example, it has been demonstrated that mammalian proteins can be linked to myristic acid by an amide-linkage to an N-terminal glycine residue, to a fatty acid or diacylglycerol through an amide- or thioether-linkage of an N-terminal cysteine, respectively, or covalently to a phophotidylinositol (PI) molecule through a C-terminal amino acid of a protein (for a review, see Biochem. J., 244:1-13, 1987). In the latter case the PI molecule is linked to the C-terminus of the protein through an intervening glycan structure, and the PI is incorporated into the phospholipid bilayer; hence the term "GPI" anchor. Specific examples of proteins know to have GPI anchors and their C-terminal amino acid sequences have been reported (Biochemica et Biophysica Acta 988:427-454, 1989; Ann. Rev. Biochem. 57:285-320, 1988). Incorporation of these amino acids or peptide domains into the amino- or carboxy-terminus of a fusion protein can direct the fusion protein to the surface of a liposome or enveloped virion.

**Preparation of Liposomes**

Liposomes are prepared by any of a variety of known techniques. For example, liposomes can be formed by any conventional technique for preparing multilamellar lipid vesicles (MLVs), i.e., by depositing one or more selected lipids on the inside wall of a suitable vessel by dissolving the lipid in chloroform, evaporating the chloroform and then adding an aqueous solution which comprises the agent(s) to be encapsulated to the vessel, allowing the aqueous solution to hydrate the lipid, and

swirling or vortexing the resulting lipid suspension. This process yields a mixture including the desired liposomes.

As another example, techniques used for producing large unilamellar vesicles (LUVs), such as, e.g., reverse-phase evaporation, infusion procedures and detergent dilution, can be used to produce the liposomes. These and other methods for producing lipid vesicles are described in Liposome Technology, Volume I (Gregoriadis, Ed., CRC Press, Boca Raton, Fla., 1984). The liposomes can be in the form of steroidal lipid vesicles, stable plurilamellar vesicles (SPLVs), monophasic vesicles (MPVs) or lipid matrix carriers (LMCs) of the type disclosed in U.S. Patents Nos. 4,588,578 and 4,610,868 (both to Fountain et al.), 4,522,803 (to Lenk et al.), and 5,008,050 (to Cullis et al.). In the case of MLVs, the liposomes can be subjected to multiple (five or more) freeze-thaw cycles to enhance their trapped volumes and trapping efficiencies and to provide a more uniform interlamellar distribution of solute if desired (Mayer et al., J. Biol. Chem., 1985, 260, 802). Specific methods for making particular oligodeoxynucleotide:liposome compositions are described in U.S. Patent No. 5,665,710 to Rahman et al.

Following their preparation, liposomes may be sized to achieve a desired size range and relatively narrow distribution of sized particles. In preferred embodiments, the liposomes have a lower range of diameters of from about 50 to about 75 nM, most preferably about 60 nM, and an upper range of diameters from about 75 to about 150 nM, most preferably about 125 nM, where "about" indicates ±10 nM.

Several techniques are available for sizing liposomes to a desired size range. Sonicating a liposome suspension by either bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles (SUVs) less than about 0.05 microns in size. Homogenization, which relies on shearing energy to fragment large liposomes into smaller ones, is another known sizing technique in which MLVs are recirculated through a standard emulsion homogenizer until a selected liposome size range, typically between about 0.1 and about 0.5 microns, is achieved. Extrusion of liposomes through a filter or membrane is another method for producing liposomes having a desired size range (see, for example, U.S. Pat. Nos. 4,737,323 to Martin et al. and 5,008,050 to Cullis et al.). Other useful sizing methods are known to those skilled in the art. In most such methods, the particle size distribution can be

monitored by conventional laser-beam size determination or other means known in the art.

Liposomes may be dehydrated, preferably under reduced pressure using standard freeze-drying equipment, for extended storage. Whether dehydrated or not, the liposomes and their surrounding media can first be frozen in liquid nitrogen and placed under reduced pressure. Although the addition of the latter freezing step makes for a longer overall dehydration process, there is less damage to the lipid vesicles, and less loss of their internal contents, when the liposomes are frozen before dehydration.

To ensure that the a significant portion of the liposomes will endure the dehydration process intact, one or more protective sugars may be made available to interact with the lipid vesicle membranes and keep them intact as water is removed. Appropriate sugars include, but are not limited to, trehalose, maltose, sucrose, lactose, glucose, dextran and the like. In general, disaccharide sugars may work better than monosaccharide sugars, with trehalose and sucrose being particularly effective in most cases, but other, more complicated sugars may alternatively be used. The amount of sugar to be used depends on the type of sugar and the characteristics of the lipid vesicles. Persons skilled in the art can readily test various sugars and concentrations to determine what conditions work best for a particular lipid vesicle preparation (see, generally, Harrigan et al., Chem. Phys. Lipids, 1990, 52, 139, and U.S. Pat. No. 4,880,635 to Janoff et al.). Generally, sugar concentrations of greater than or equal to about 100 mM have been found to result in the desired degree of protection. Once the liposomes have been dehydrated, they can be stored for extended periods of time until they are to be used. The appropriate conditions for storage will depend on the chemical composition of the lipid vesicles and their encapsulated active agent(s). For example, liposomes comprising heat labile agents should be stored under refrigerated conditions so that the potency of the active agent is not lost.

Cationic liposomes are useful for gene transfer purposes. DNA, nucleic acids, and/or oligonucleotides may be loaded into cationic liposomes for delivery into cells. The use of cationic lipids may alter the infective range of a virion, e.g., lessen the virion's need for specific receptors for cellular attachment (Innes et al., Cationic

liposomes (Lipofectin) mediate retroviral infection in the absence of specific receptors J. Virol. 64:957-961, 1990).

Cationic liposomes may, by way of non-limiting example, comprise dioleyloxypropyl-trimethylammonium (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE) (Lipofectin, BRL) and used for transfection (Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., and Danielsen, M. (1987) Lipofection: A highly efficient, lipid-mediated DNA transfection procedure. Proc. Natl. Acad. Sci. USA, 84:7413-7417; Gao, X and Huang, L. (1995) Cationic liposome-mediated gene transfer. Gene Therapy, 2:710-722). DOPE is often called a helper lipid.

A cationic lipid is comprised of a lipid anchor, a linker bond, a spacer, and a head group. The lipid anchor contrains the biophysical properties (rigidity, rate of exchange of lipid molecules between lipid membranes, etc.) of the bilayer, cholesterol and fatty acid chains are typical anchors used for this purpose. How the linker is bonded constrains the ability of the cationic lipid to be degraded and the stability of the cationic lipid. The spacer between the anchor and the linker bond and the head group can vary between reasonable limits. The nature of the head groups of the cationic lipid is important to determine the overall transfection activity and to control the cytotoxicity of the cationic lipid. Lipids with quaternary amine groups (ammonium group) have been properties in condensing DNA compared to tertiary amine groups. Cationic lipids are described by Gao, X and Huang, L. (1995) Cationic liposome-mediated gene transfer. Gene Therapy, 2:710-722; .Farhood, H., Serbina, N., and Huang, L.,(1995) The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim. Biophys. Acta 1235:289-295; Sternberg, B., Sorgi, F.L., and Huang, L. (1994) New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy. FEBS Lett. 356: 361-366; Zhou, X. and Huang, L. (1994) DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. Biochim. Biophys. Acta. 1189:195-203).

**Pharmaceutical Formulations of Liposomes**

Numerous pharmaceutical formulations of liposomes have been developed for delivery to a variety of cell types and tissues have been described. Non-limiting

examples include formulations for the intranasal administration of vaccines (U.S. Patent 5,756,104), and aerosol formulations for the delivery of anti-cancer drugs (U.S. Patent 6,090,407). Liposomes may be encapsulated by, and/or incorporated into, formulations such as pills, tablets, capsules, caplets, suppositories, liquids designed for deliver via the alimentary canal, preferably via oral administartion. Pharmaceutical formulations that comprise liposomes and which are used for the delivery of macromolecules, including but not limited to proteins and nucleic acids, are described in, by way of non-limiting example, U.S. Patents 6,132,764, Targeted polymerized liposome diagnostic and treatment agents; 5,879,713, Targeted delivery via biodegradable polymers; 5,851,548, Liposomes containing cationic lipids and vitamin D; 5,759,519, Method for the intracellular delivery of biomolecules using thiocationic lipids; 5,756,352, Thiocationic lipid-nucleic acid conjugates; 5,739,271, Thiocationic lipids; 5,711,964, Method for the intracellular delivery of biomolecules using liposomes containing cationic lipids and vitamin D; and 5,494,682, Ionically cross-linked polymeric microcapsules.

Liposomal gene delivery vectors have been used for the delivery of biologically active genes, including by way of non-limiting example, the delivery of alpha-1-antitrypsin, and the cystic fibrosis conductance regulator (CFTR) through aerosol administration of DNA/lipid complexes. Additional animal studies include experimental tumor model central nervous system and arterial wall. Literature describing these results are herein incorporated by reference (Carnonico, A., Conary, J., Christman, B., Meyrick, B., and Brigam K.L. (1994) Aerosol and intravenous transfection of huamn alpha-1-antitrypsin gene to lungs of rabbits. Am. J. Respir.Cell Mol. Biol. 10:24-29.; Stewart, M.J., Plautz, G.E., Buonp, L.D., Yang, Z.Y., Xu, L., Gao, X., Huang, L., Nabel, E.G., and Nabel, G.J. (1992) Gene transfer in vivo with DNA-liposome complexes : Safety and acute oxicity in mice. Human Gene Therapy 3: 267-275; Ono, T., Fujino, Y., Tsuchiya, T., and Tsuda, M. (1990) Plasmid DNAs dierctly injected into mouse brain with lipofectin can be incorporated and expressed by mouse brain with lipofectin can be incorporated and expressed by brain cells. Neurosci. Lett. 117, 259-263; Jiao, S., Acsadi, G., Jani, A., Felgner, P., Wolff, J.A.(1992) Persistence of plasmid DNA and expression in rat brain cells in vivo. Exp.Neurobiol.115, 400-413; Nabel, E.G., Plautz, G., Boyce, F.M., Stanley,

J. C., Nabel, G.L. (1989) Recombinant gene expression in vivo within endothelial cells of the arterial wall. Science 244, 1342-1343; Nabel, G.L., Nabel, E.G., Yang, Z, Fox, B.A., Plautz, G.E., Gao, X., Huang, L., Shu, S., Gordon, D., Chang, A.E., (1993) Direct gene transfer with DNA liposome complexes in melanoma: Expression, biological activity, lack of toxicity in humans. Proc. Natl. Acad. Sci. USA, 90,11307-11311; Caplen, N.J., Alton, E.F.W.W., Middleton, P.G., Dorin, I.R., Stevenson, B.J., Gao, X., Durham, S.R., Jeffery, P., K., Hodson, M.E., Coutelle, C., Huang, L., Porteous, D.J., Williamson, R., Geddes, D.M.(1994) Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. Nature Medicine 1, 39-46).

## VI. ANTIBODIES AND ANTIBODY DERIVATIVES

The term "antibody" is meant to encompass an immunoglobulin molecule obtained by in vitro or in vivo generation of an immunogenic response, and includes both polyclonal, monospecific and monoclonal antibodies. An "immunogenic response" is one that results in the production of antibodies directed to one or more proteins after the appropriate cells have been contacted with such proteins, or polypeptide derivatives thereof, in a manner such that one or more portions of the protein function as epitopes. An epitope is a single antigenic determinant in a molecule. In proteins, particularly denatured proteins, an epitope is typically defined and represented by a contiguous amino acid sequence. However, in the case of nondenatured proteins, epitopes also include structures, such as active sites, that are formed by the three-dimensional folding of a protein in a manner such that amino acids from separate portions of the amino acid sequence of the protein are brought into close physical contact with each other.

Wildtype antibodies have four polypeptide chains, two identical heavy chains and two identical light chains. Both types of polypeptide chains have constant regions, which do not vary or vary minimally among antibodies of the same class (i.e, IgA, IgM, etc.), and variable regions. As is explained below, variable regions are unique to a particular antibody and comprise a recognition element for an epitope.

Each light chain of an antibody is associated with one heavy chain, and the two chains are linked by a disulfide bridge formed between cysteine residues in the

carboxy-terminal region of each chain, which is distal from the amino terminal region of each chain that constitutes its portion of the antigen binding domain. Antibody molecules are further stabilized by disulfide bridges between the two heavy chains in an area known as the hinge region, at locations nearer the carboxy terminus of the heavy chains than the locations where the disulfide bridges between the heavy and light chains are made. The hinge region also provides flexibility for the antigen-binding portions of an antibody.

An antibody's specificity is determined by the variable regions located in the amino terminal regions of the light and heavy chains. The variable regions of a light chain and associated heavy chain form an "antigen binding domain" that recognizes a specific epitope; an antibody thus has two antigen binding domains. The antigen binding domains in a wildtype antibody are directed to the same epitope of an immunogenic protein, and a single wildtype antibody is thus capable of binding two molecules of the immunogenic protein at the same time.

Compositions of antibodies have, depending on the manner in which they are prepared, different types of antibodies. Types of antibodies of particular interest include polyclonal, monospecific and monoclonal antibodies.

Polyclonal antibodies are generated in a immunogenic response to a protein having many epitopes. A composition of polyclonal antibodies thus includes a variety of different antibodies directed to the same and to different epitopes within the protein. Methods for producing polyclonal antibodies are known in the art (see, e.g., Cooper et al., Section III of Chapter 11 in: Short Protocols in Molecular Biology, 2nd Ed., Ausubel et al., eds., John Wiley and Sons, New York, 1992, pages 11-37 to 11-41).

Monospecific antibodies (a.k.a. antipeptide antibodies) are generated in a humoral response to a short (typically, 5 to 20 amino acids) immunogenic polypeptide that corresponds to a few (preferably one) isolated epitopes of the protein from which it is derived. A plurality of monospecific antibodies includes a variety of different antibodies directed to a specific portion of the protein, i.e, to an amino acid sequence that contains at least one, preferably only one, epitope. Methods for producing monospecific antibodies are known in the art (see, e.g., Cooper et al., Section III of

Chapter 11 in: Short Protocols in Molecular Biology, 2nd Ed., Ausubel et al., eds., John Wiley and Sons, New York, 1992, pages 11-42 to 11-46).

A monoclonal antibody is a specific antibody that recognizes a single specific epitope of an immunogenic protein. In a plurality of a monoclonal antibody, each antibody molecule is identical to the others in the plurality. In order to isolate a monoclonal antibody, a clonal cell line that expresses, displays and/or secretes a particular monoclonal antibody is first identified; this clonal cell line can be used in one method of producing the antibodies of the invention. Methods for the preparation of clonal cell lines and of monoclonal antibodies expressed thereby are known in the art (see, for example, Fuller et al., Section II of Chapter 11 in: Short Protocols in Molecular Biology, 2nd Ed., Ausubel et al., eds., John Wiley and Sons, New York, 1992, pages 11-22 to 11-11-36).

Variants and derivatives of antibodies include antibody and T-cell receptor fragments that retain the ability to specifically bind to antigenic determinants. Preferred fragments include Fab fragments (i.e, an antibody fragment that contains the antigen-binding domain and comprises a light chain and part of a heavy chain bridged by a disulfide bond); Fab' (an antibody fragment containing a single anti-binding domain comprising an Fab and an additional portion of the heavy chain through the hinge region); F(ab')2 (two Fab' molecules joined by interchain disulfide bonds in the hinge regions of the heavy chains; the Fab' molecules may be directed toward the same or different epitopes); a bispecific Fab (an Fab molecule having two antigen binding domains, each of which may be directed to a different epitope); a single chain Fab chain comprising a variable region, a.k.a., a sFv (the variable, antigen-binding determinative region of a single light and heavy chain of an antibody linked together by a chain of 10-25 amino acids); a disulfide-linked Fv, or dsFv (the variable, antigen-binding determinative region of a single light and heavy chain of an antibody linked together by a disulfide bond); a camelized VH (the variable, antigen-binding determinative region of a single heavy chain of an antibody in which some amino acids at the VH interface are those found in the heavy chain of naturally occurring camel antibodies); a bispecific sFv (a sFv or a dsFv molecule having two antigen-binding domains, each of which may be directed to a different epitope); a diabody (a dimerized sFv formed when the VH domain of a first sFv assembles with

the VL domain of a second sFv and the VL domain of the first sFv assembles with the VH domain of the second sFv; the two antigen-binding regions of the diabody may be directed towards the same or different epitopes); and a triabody (a trimerized sFv, formed in a manner similar to a diabody, but in which three antigen-binding domains are created in a single complex; the three antigen binding domains may be directed towards the same or different epitopes). Derivatives of antibodies also include one or more CDR sequences of an antibody combining site. The CDR sequences may be linked together on a scaffold when two or more CDR sequences are present.

The term "antibody" also includes genetically engineered antibodies and/or antibodies produced by recombinant DNA techniques and "humanized" antibodies. Humanized antibodies have been modified, by genetic manipulation and/or in vitro treatment to be more human, in terms of amino acid sequence, glycosylation pattern, etc., in order to reduce the antigenicity of the antibody or antibody fragment in an animal to which the antibody is intended to be administered (Gussow et al., Methods Enz. 203:99-121, 1991).

**Methods of Preparing Antibodies and Antibody Variants**

The antibodies and antibody fragments of the invention may be produced by any suitable method, for example, in vivo (in the case of polyclonal and monospecific antibodies), in cell culture (as is typically the case for monoclonal antibodies, wherein hybridoma cells expressing the desired antibody are cultured under appropriate conditions), in in vitro translation reactions, and in recombinant DNA expression systems (the latter method of producing proteins is described in more detail herein in the section entitled "Methods of Producing Fusion Proteins"). Antibodies and antibody variants can be produced from a variety of animal cells, preferably from mammalian cells, with murine and human cells being particularly preferred. Antibodies that include non-naturally occurring antibody and T-cell receptor variants that retain only the desired antigen targeting capability conferred by an antigen binding site(s) of an antibody can be produced by known cell culture techniques and recombinant DNA expression systems (see, e.g., Johnson et al., Methods in Enzymol. 203:88-98, 1991; Molloy et al., Mol. Immunol. 32:73-81, 1998; Schodin et al., J. Immunol. Methods 200:69-77, 1997). Recombinant DNA expression

systems are typically used in the production of antibody variants such as, e.g., bispecific antibodies and sFv molecules. Preferred recombinant DNA expression systems include those that utilize host cells and expression constructs that have been engineered to produce high levels of a particular protein. Preferred host cells and expression constructs include Escherichia coli; harboring expression constructs derived from plasmids or viruses (bacteriophage); yeast such as Sacharomyces cerevisieae or Fichia pastoras harboring episomal or chromosomally integrated expression constructs; insect cells and viruses such as Sf 9 cells and baculovirus; and mammalian cells harboring episomal or chromosomally integrated (e.g., retroviral) expression constructs (for a review, see Verma et al., J. Immunol. Methods 216:165-181, 1998). Antibodies can also be produced in plants (U.S. Patent 6,046,037; Ma et al., Science 268:716-719, 1995) or by phage display technology (Winter et al., Annu. Rev. Immunol. 12:433-455, 1994).

XenoMouse strains are genetically engineered mice in which the murine IgH and Igk locci have been functionally replaced by their Ig counterparts on yeast artificial YAC transgenes. These human Ig transgenes can carry the majority of the human variable repertoire and can undergo class switching from IgM to IgG isotypes. The immune system of the xenomouse recognizes administered human antigens as foreign and produces a strong humoral response. The use of XenoMouse in conjunction with well-established hybridomas techniques, results in fully human IgG mAbs with sub-nanomolar affinities for human antigens (for a review, see Green, J. Immunol. Methods 231:11-23, 1999).

The single-chain antibody sFv-5A, and derivatives thereof such as sFv-5AF, is used in competition assays to identify molecules that prevent the binding of sFv-5A to this region of the polyimmunoglobulin receptor (pIgR) and/or the pIgR stalk protein, or derivatives or conjugates thereof.

The sFv-5A compound is a non-limiting example of a sFv (or antibodies and fragments derived therefrom) that may be used as a pIgR targeting element in compounds that are intended to undergo endocytosis, transcytosis and/or exocytosis across an epithelial surface. Any sFv that reacts with the pIgR stalk molecule or transcytotic molecule is used to deliver macromolecules into, through and out of cell,

especially epithelial cells, but some may have better binding and transport features than others.

Anti-pIgR sFvs are characterized with respect to their epitope binding sites. However, some sFv molecules may not react with pIgR in such a way as to identify a linear epitope, because the eptiope that is recognized may be comprised of regions of amino acid sequence that are remote in the linear sequence of pIgR. Reduced reactivity or no reactivity at all with a nest set of oligopeptides is usually achieved with 'non-linear' or 'conformational' epitopes.

These and other sFvs are tagged with antigenic peptides, such as myc and flag, to which commercially available antibodies and antibody-conjugates are available. The tag sequence is placed on the amino terminus or the carboxy terminus of the sFv and is separated by a flexible tether, such as (Gly4Ser)x, where x is 1 to 4 and preferably 2 to 3. Tagged sFv is quantitated by reaction with antibody-conjugates that are detectable. Detection is achieved by using horse radish peroxidase or alkaline phosphate or other detectable systems attached to the antibody that has specificity for the tag.

## VII. PHARMACEUTICAL COMPOSITIONS AND THERAPEUTIC METHODS

Another aspect of the invention is drawn to compositions, including but not limited to pharmaceutical compositions. According to the invention, a "composition" refers to a mixture comprising at least one carrier, preferably a physiologically acceptable carrier, and one or more pIgR-targeting membrane-bounded vesicles and virions. The term "carrier" defines a chemical compound that does not inhibit or prevent the incorporation of the biologically active peptide(s) into cells or tissues. A carrier typically is an inert substance that allows an active ingredient to be formulated or compounded into a suitable dosage form (e.g., a pill, a capsule, a gel, a film, a tablet, a microparticle (e.g., a microsphere), a solution; an ointment; a paste, an aerosol, a droplet, a colloid or an emulsion etc.). A "physiologically acceptable carrier" is a carrier suitable for use under physiological conditions that does not abrogate (reduce, inhibit, or prevent) the biological activity and properties of the compound. For example, dimethyl sulfoxide (DMSO) is a carrier as it facilitates the

uptake of many organic compounds into the cells or tissues of an organism. Preferably, the carrier is a physiologically acceptable carrier, preferably a pharmaceutically or veterinarily acceptable carrier, in which the chimeric pIgR-targeting protein is disposed.

A "pharmaceutical composition" refers to a composition wherein the carrier is a pharmaceutically acceptable carrier, while a "veterinary composition" is one wherein the carrier is a veterinarily acceptable carrier. The term "pharmaceutically acceptable carrier" or "veterinarily acceptable carrier" includes any medium or material that is not biologically or otherwise undesirable, i.e, the carrier may be administered to an organism along with a chimeric pIgR-targeting protein conjugate, composition or compound without causing any undesirable biological effects or interacting in a deleterious manner with the complex or any of its components or the organism. Examples of pharmaceutically acceptable reagents are provided in The United States Pharmacopeia, The National Formulary, United States Pharmacopeial Convention, Inc., Rockville, Md. 1990, hereby incorporated by reference herein into the present application. The terms "therapeutically effective amount" or "pharmaceutically effective amount" mean an amount sufficient to induce or effectuate a measurable response in the target cell, tissue, or body of an organism. What constitutes a therapeutically effective amount will depend on a variety of factors which the knowledgeable practitioner will take into account in arriving at the desired dosage regimen.

The compositions of the invention can further comprise other chemical components, such as diluents and excipients. A "diluent" is a chemical compound diluted in a solvent, preferably an aqueous solvent, that facilitates dissolution of the therapeutic agent in the solvent, and it may also serve to stabilize the biologically active form of the chimeric pIgR-targeting protein or one or more of its components. Salts dissolved in buffered solutions are utilized as diluents in the art. For example, preferred diluents are buffered solutions containing one or more different salts. A preferred buffered solution is phosphate buffered saline (particularly in conjunction with compositions intended for pharmaceutical administration), as it mimics the salt conditions of human blood. Since buffer salts can control the pH of a solution at low

concentrations, a buffered diluent rarely modifies the biological activity of a biologically active peptide.

An "excipient" is any more or less inert substance that can be added to a composition in order to confer a suitable property, for example, a suitable consistency or to form a drug. Suitable excipients and carriers include, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol cellulose preparations such as, for example, maize starch, wheat starch, rice starch, agar, pectin, xanthan gum, guar gum, locust bean gum, hyaluronic acid, casein potato starch, gelatin, gum tragacanth, polyacrylate, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents can also be included, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Other suitable excipients and carriers include hydrogels, gellable hydrocolloids, and chitosan. Chitosan microspheres and microcapsules can be used as carriers. See WO 98/52547 (which describes microsphere formulations for targeting compounds to the stomach, the formulations comprising an inner core (optionally including a gelled hydrocolloid) containing one or more active ingredients, a membrane comprised of a water insoluble polymer (e.g., ethylcellulose) to control the release rate of the active ingredient(s), and an outer layer comprised of a bioadhesive cationic polymer, for example, a cationic polysaccharide, a cationic protein, and/or a synthetic cationic polymer; U.S. patent No. 4,895,724. Typically, chitosan is cross-linked using a suitable agent, for example, glutaraldehyde, glyoxal, epichlorohydrin, and succinaldehyde. Compositions employing chitosan as a carrier can be formulated into a variety of dosage forms, including pills, tablets, microparticles, and microspheres, including those providing for controlled release of the active ingredient(s). Other suitable bioadhesive cationic polymers include acidic gelatin, polygalactosamine, polyamino acids such as polylysine, polyhistidine, polyornithine, polyquaternary compounds, prolamine, polyimine, diethylaminoethyldextran (DEAE), DEAE-imine, DEAE-methacrylate, DEAE-acrylamide, DEAE-dextran, DEAE-cellulose, poly-p-aminostyrene, polyoxethane, copolymethacrylates, polyamidoamines, cationic starches, polyvinylpyridine, and polythiodiethylaminomethylethylene.

The compositions of the invention can be formulated in any suitable manner. The pIgR-targeting membrane-bounded vesicles and virions therein may be uniformly (homogeneously) or non-uniformly (heterogenously) dispersed in the carrier. Suitable formulations include dry and liquid formulations. Dry formulations include freeze dried and lyophilized powders, which are particularly well suited for aerosol delivery to the sinuses or lung, or for long term storage followed by reconstitution in a suitable diluent prior to administration. Other preferred dry formulations include those wherein a composition according to the invention is compressed into tablet or pill form suitable for oral administration or compounded into a sustained release formulation. When the composition is intended for oral administration but the pIgR-targeting membrane-bounded vesicles and virions are to be delivered to epithelium in the intestines, it is preferred that the formulation be encapsulated with an enteric coating to protect the formulation and prevent premature release of the pIgR-targeting membrane-bounded vesicles and virions included therein. As those in the art will appreciate, the compositions of the invention can be placed into any suitable dosage form. Pills and tablets represent some of such dosage forms. The compositions can also be encapsulated into any suitable capsule or other coating material, for example, by compression, dipping, pan coating, spray drying, etc. Suitable capsules include those made from gelatin and starch. In turn, such capsules can be coated with one or more additional materials, for example, and enteric coating, if desired. Liquid formulations include aqueous formulations, gels, and emulsions.

Some preferred embodiments concern compositions that comprise a bioadhesive, preferably a mucoadhesive, coating. A "bioadhesive coating" is a coating that allows a substance (e.g., a composition or pIgR-targeting membrane-bounded vesicles and virions according to the invention) to adhere to a biological surface or substance better than occurs absent the coating. A "mucoadhesive coating" is a preferred bioadhesive coating that allows a substance, for example, a composition according to the invention, to adhere better to mucosa occurs absent the coating. For example, micronized particles (e.g., particles having a mean diameter of about 5, 10, 25, 50, or 100 μm) can be coated with a mucoadhesive. The coated particles can then be assembled into a dosage form suitable for delivery to an organism. Preferably, and depending upon the location where the cell surface

transport moiety to be targeted is expressed, the dosage form is then coated with another coating to protect the formulation until it reaches the desired location, where the mucoadhesive enables the formulation to be retained while the chimeric pIgR-targeting proteins interact with the target cell surface transport moiety.

The compositions of the invention facilitate administration of chimeric pIgR-targeting membrane-bounded vesicles and virions to an organism, preferably an animal, preferably a mammal, bird, fish, insect, or arachnid. Preferred mammals include bovine, canine, equine, feline, ovine, and porcine animals, and non-human primates. Humans are particularly preferred. Multiple techniques of administering or delivering a compound exist in the art including, but not limited to, oral, rectal (e.g. an enema or suppository) aerosol (e.g., for nasal or pulmonary delivery), parenteral, and topical administration. Sufficient quantities of the biologically active component are delivered to achieve the intended effect. The particular amount of biologically active component to be delivered will depend on many factors, including the effect to be achieved, the type of organism to which the composition is delivered, delivery route, dosage regimen, and the age, health, and sex of the organism. As such, the particular dosage of chimeric pIgR-targeting membrane-bounded vesicles and virions included in a given formulation is left to the ordinarily skilled artisan's discretion.

Those skilled in the art will appreciate that when the compositions of the present invention are administered as agents to achieve a particular desired biological result, which may include a therapeutic or protective effect(s) (including vaccination), it may be necessary to combine the fusion proteins of the invention with a suitable pharmaceutical carrier. The choice of pharmaceutical carrier and the preparation of the fusion protein as a therapeutic or protective agent will depend on the intended use and mode of administration. Suitable formulations and methods of administration of therapeutic agents include those for oral, pulmonary, nasal, buccal, ocular, dermal, rectal, or vaginal delivery.

Depending on the mode of delivery employed, the context-dependent functional entity can be delivered in a variety of pharmaceutically acceptable forms. For example, the context-dependent functional entity can be delivered in the form of a solid, solution, emulsion, dispersion, micelle, liposome, and the like, incorporated

into a pill, capsule, tablet, suppository, areosol, droplet, or spray. Pills, tablets, suppositories, areosols, powders, droplets, and sprays may have complex, multilayer structures and have a large range of sizes. Aerosols, powders, droplets, and sprays may range from small (1 micron) to large (200 micron) in size.

Pharmaceutical compositions of the present invention can be used in the form of a solid, a lyophilized powder, a solution, an emulsion, a dispersion, a micelle, a liposome, and the like, wherein the resulting composition contains one or more of the compounds of the present invention, as an active ingredient, in admixture with an organic or inorganic carrier or excipient suitable for enteral or parenteral applications. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use. The carriers which can be used include glucose, lactose, mannose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, medium chain length triglycerides, dextrans,and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form. In addition auxiliary, stabilizing, thickening and coloring agents and perfumes may be used. Examples of a stabilizing dry agent includes triulose, preferably at concentrations of 0.1% or greater (See, e.g., U.S. Patent No. 5,314,695).

Pharmaceutical formulations of particular interest in the context of the invention include, but are not limited to, those taught is U.S. Patents Nos. 5,254,342, entitled "Compositions and methods for enhanced transepithelial and transendothelial transport o[f] active agents" to Shen et al.; and 6,110,456, "Oral delivery o[f] adeno-associated viral vectors."

**Uses of Compositions**

The invention's compositions facilitate administration of pIgR-targeting membrane-bounded vesicles and virions to an organism, preferably an animal, preferably a mammal, bird, fish, insect, or arachnid. Preferred mammals include bovine, canine, equine, feline, ovine, and porcine animals, and non-human primates. Humans are particularly preferred. Multiple techniques of administering or delivering a compound exist in the art including, but not limited to, oral, aerosol

(e.g., for nasal or pulmonary delivery), parenteral, and topical administration. Sufficient quantities of the biologically active component are delivered to achieve the intended effect. The particular amount of biologically active component to be delivered will depend on many factors, including the effect to be achieved, the type of organism to which the composition is delivered, delivery route, dosage regimen, and the age, health, and sex of the organism. As such, the particular dosage of pIgR-targeting membrane-bounded vesicles and virions to be included in a given formulation is left to the ordinarily skilled artisan's discretion.

Thus, another aspect of the invention relates to delivering a composition according to the invention to an organism. As a result, pIgR-targeting membrane-bounded vesicles and virions of the composition are delivered to cells expressing the pIgR protein. The pIgR expressing cell of the present invention is preferably a mammalian cell and more preferably a mammalian epithelial cell that normally secretes IgA. Such epithelial cells that normally secrete IgA can be found in the intestinal tract, the oral cavity, the nasal cavity, the respiratory tract, the ocular surfaces, and the dermal surfaces of a mammal.

A related aspect concerns various applications for the pIgR-targeting membrane-bounded vesicles and virions and compositions of the invention. These include prophylactic and therapeutic applications. For example, a preferred prophylactic application is vaccination, wherein a composition according to the invention allows for the introduction of a polypeptide antigen, or a nucleic acid encoding an antigen. The composition is delivered and elicits an immune response, preferably a protective immune response, in the organism to which the composition was administered. In general, the vaccines of the invention are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per individual. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial

administration followed in one or two week intervals by a subsequent injection or other administration.

In another therapeutic context, the pIgR-targeting membrane-bounded vesicles and virions and compositions allow a biologically active peptide having a therapeutic effect to be efficaciously delivered as part of a pIgR-targeting protein conjugate. Because pIgR-targeting membrane-bounded vesicles and virions are delivered into cells by active transport, the instant compositions afford better control over bioavailability of biologically active peptides, as compared to passive transport mechanisms. As such, the pIgR-targeting membrane-bounded vesicles and virions and compositions of the invention enable improved uptake and utilization of the biologically active peptide.

The membrane-bounded vesicles and virions of the invention are also useful in diagnostic and related applications. Another aspect of the invention is compositions and methods comprising the membrane-bounded vesicles and virions of the invention for the diagnosis and monitoring of certain diseases and disorders, preferably in kit form. This aspect is useful for assaying and monitoring the course of the diagnosis and prognosis of disease, for monitoring the effectiveness and/or distribution of a therapeutic agent or an endogenous protein, in a patient as well as other related functions.

In this aspect of the invention, it may be desirable to monitor or determine if, or determine the degree to which, a patient's pIgR-displaying cells are capable of, or presently are, endocytosing a detectably labeled fusion protein of the invention. Such methods are used in a variety of systems depending on the nature of the pIgR-targeting element(s) of a given protein conjugate.

For example, the degree to which a patient, or a biological sample therefrom, endocytoses a membrane-bounded vesicle or virion displaying a pIgR-targeting element derived from a bacterial protein that binds pIgR is a measure of a patient's susceptibility to infection by bacteria having that element. A higher degree or rate of uptake of the detectably labeled protein conjugate indicates that the patient is more susceptible to such infection.

As another example, the activity, distribution and/or concentration of endogenous pIgR proteins may be altered in various ways during the course of a

disease or disorder. The pIgR-targeted membrane-bounded vesicles and virions in a patient are measured over the course of a disease for diagnostic and prognostic purposes, as well as over the course of treatment of a disease or disorder, in order to monitor the effects on pIgR proteins. Diseases to which this aspect of the invention can be applied include but are not limited to diseases that involve the respiratory system, such as lung cancer and tumors, asthma, pathogenic infections, allergy-related disorders, and the like; the gastrointestinal tract, including cancers, tumors, pathogenic infections, disorders relating to gastroinstestinal hormones, Chron's disease, eating disorders, and the like; and any disease or disorder that is known or suspected to involve pIgR-displaying cells.

Polypeptides per se and the expression of nucleic acids encoding polypeptides may be detected by virute of comprising a detectable polypeptide such as, e.g., a green fluorescent protein (GFP) or a derivative thereof, or luciferase (Peters et al., Optimization of cationic liposome-mediated gene transfer to human bronchial epithelial cells expressing wild-type or abnormal cystic fibrosis transmembrane conductance regulator (CFTR), Exp Lung Res 1999 Apr-May;25(3):183-97; Oudhiri et al., Proc. Natl. Acad. Sci. USA 94:1651-1656, 1997). Confocal microscopy may be used to follow GTA delivery (Serafino et al., Cellular uptake and delivery monitoring of liposome/DNA complexes during in vitro transfection of CFTR gene, Biochem Mol Biol Int 1999 Feb;47(2):337-44). If the membrane-bounded vesicle or virion comprises an epitope for which antibodies are available (including but nor limited to commercially avalable ones such as c-myc epitope and the FLAG-tag), it may be detected using any of a variety of immunoassays such as enzyme-linked immunosorbent assay (ELISA) or a radioimmunoassay (RIA). PCR may also be used to evaluate gene delivery vectors (Rettinger et al., Proc. Natl. Acad. Sci. USA 91:1460-1464, 1994).

The contents of the articles, patent, and patent applications, and all other documents and electronically available information mentioned or cited herein, are hereby incorporated by reference in their entirety to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. Applicants reserve the right to physically incorporate into this

application any and all materials and information from any such articles, patents, patent applications or other documents.

## EXAMPLES

### EXAMPLE 1: ANTIBODIES DIRECTED TO PIGR MOLECULES

**ELISA for Monoclonal Antibodies**

An assay is prepared by applying purified pIgR stalk molecules or GST-pIgR stalk molecules, or any other pIgR target, to multiwell (48-well, 96-well and other size plates and allowing the protein to adhere to the wells of the plates during overnight incubation. The plates are washed to remove unbound proteins. Samples of the serum from the immunized mice are incubated with the pIgR or GST-pIgR coated plates. After 1 to 2 hours of incubation (gentle shaking at room temperature), the plate is washed free of unreacted immune serum proteins. Mouse antibodies that react with an immobilized GST-pIgR protein are detected by adding to each well a sample of a goat antibody that has been raised against and is directed to mouse immunoglobulin, i.e., all subclasses of murine immunoglobulins. The goat antibody is conjugated to an enzyme that is used for detection; non-limiting examples include horse radish peroxidase and alkaline phosphatase. After unreacted horse radish peroxidase or alkaline phosphatase conjugated goat anti-mouse immunoglobulin has been washed from the wells, the substrate of horse radish peroxidase or alkaline phosphatase is added. When the color is sufficiently developed, the reaction is stopped and quantitated using a spectrophotometer. In the positive wells, antibodies against the GST-pIgR protein will be present. Some of these antibodies are directed to the GST portion of the protein if GST-pIgR is used. By assaying against other GST fusion proteins, it is determined if the antibodies are against GST or pIgR. This assay is also used to identify antibody producing cells and clones in 96-well plates that are part of the process of isolating clones of hybridomas that produce the desired monoclonal antibody.

Beads that bind GST moieties on GST-fusion proteins are also used for assays. GST-pIgR bound to beads is reacted with sera that contain antibodies directed against pIgR. The antibodies that react with and bind to pIgR can then be detected by an anti-antibody conjugated to horse radish peroxidase or alkaline phosphatase. If the

antibodies that react with pIgR are derived from mice, then the antibodies that detected the presence of the mouse antibody is obtained from another animal species, such as goat or sheep. Those skilled in the art will know how to adjust the source and specificity of the detecting antibody conjugates (i.e. horse radish peroxidase or alkaline phosphatase conjugated to anti-FLAG tag antibody) to obtain the desired results.

**Preparation of Monoclonal Antibodies (Mabs)**

Monoclonal antibodies are created by immunizing mice with portions of pIgR, generally prepared as oligopeptides having defined amino acid sequences. For example, a nucleic acid encoding an amino acid sequence found in a conserved region of pIgR, such as those described in Table 2, or a amino acid sequence that varies between homolgs, such as, e.g., R1, R2a, R2b, R3a, R3b, R3c (etc.) is used to create a pIgR-target-GST fusion protein that is expressed in a host cell such as E. coli. The GST portion of the fusion protein is used to isolate the fusion protein, and the purified GST-pIgR protein is mixed with adjuvant and injected into mice to produce an immune response. The extent of the immune response is measured over time by removing blood from the immunized mice at regular intervals and measuring the level of antibodies directed to the GST-pIgR fusion protein using an immunoassay, e.g., an ELISA.

Once the immunized mouse has been shown to be producing antibodies directed to the GST-pIgR fusion protein, the spleen of the mouse is harvested, and cells therefrom are prepared for fusion with immortalized fusion partners, such as the NS/1 cell line, according to Kohler and Milstein, in order to create Mab-producing hybridoma cell lines. Independently isolated clones and subclones are grown to an appropriate density, the cell supernatant is assayed using an ELISA to determine if antibodies that react with the GST-pIgR fusion proteins are produced by each clone or subclone. Positive wells are assayed using limiting dilution, and clonal and subclonal cell lines are eventually obtained that produce Mabs against either the GST-pIgR fusion protein.

By assaying and comparing results from assays using commercially available monoclonal antibodies directed to GST, and GST fusion proteins that do not contain pIgR, as well as polyclonal antibodies directed to pIgR, it is possible to identify

isolated Mabs that either are pIgR specific or are specific to an epitope not present in either pIgR or GST but which occurs at the junction thereof. The Mabs can additionally be tested for specificity using MDCK cells and MDCK cells that have been transfected with different species of pIgR (human, rat, mouse, pig, rabbit, monkey, etc.).

A collection of monoclonal antibodies and sFvs that cumulatively bind to many, preferably every, epiotpe of pIgR domain 6, which includes the pIgR stalk, is prepared. Each of the sFvs and the Mabs are epitope mapped using the nested set of overlapping oligopeptides (each comprising 5 to 20 amino acids). Linear epitopes and conformational epitopes are identified on the strength of their binding and the location of the peptides in the nested set.

**Single-Chain Antibodies to pIgR Molecules**

A useful pIgR ligand that may be used as a reagent is an antibody directed to pIgR, or an active fragment or derivative of such an antibody. Any sFv that binds to pIgR may be used in the methods to identify small molecules that bind to pIgR.

As one example of a useful sFv, sFv-5A is human sFv that recognizes an epitope on human and rat polymeric immunoglobulin receptor (pIgR). See Figure 3. The sFv-5A compound is described in U.S. patent application Serial No. 60/192,197 (attorney docket no. 18062E-000900 entitled "Ligands Directed to the Non-Secretory Component, Non-Stalk Region of pIgR and Methods of Use Thereof" by Mostov, Keith E., and Chapin, Steven J.), filed March 27, 2000, describes the B region of pIgR and ligands directed to the B region of pIgR; and U.S. patent application Serial No. 60/192,198 (attorney docket no. 18062E-003000US entitled "Anti-pIgR Antibodies With Improved Transcytosis by Mostov, Keith E., Chapin, Steven J., and Richman-Eisenstat, Janice), filed March 27, 2000.

Another type of sFv of note is one that binds the secretory component (SC) and is thus useful to distinguish between ligands that bind that molecule and those that bind the pIgR stalk molecule. One example of an SC-directed sFv is taught in U.S. Patent 6,072,041.

## EXAMPLE 2: PIGR-TARGETING ELEMENTS DERIVED FROM BACTERIAL PROTEINS

Published studies by Zhang et al. (Cell 102:827-837, 2000) indicate that pIgR binds to or interacts with bacteria to provide a mechanism which provides bacterial cells enhanced abilities to adhere, invade, and undergo apical to basolateral transcytotic migration. The studies provide evidence that there are pIgR-binding regions on the surface of bacteria. Within the scope of the present invention, various pIgR-binding regions are provided that are derived from surface proteins of bacteria.

The pneumococcal adhesin protein CpbA interacts with human pIgR (hpIgR) as either a part of the outer surface of a bacterial cell or as a free molecule. The regions of CpbA:hpIgR interaction were mapped using a series of large peptide fragments derived from CpbA. CpbA (Swiss-Prot Accession No. O30874) contains a choline binding domain containing residues 454-663 and two N-terminal repetitive regions called R1 and R2 that are contained in residues 97-203 and 259-365, respectively. Zhang et al. demonstrated that polypeptides containing R1 (107 amino acid residues) and R2 (see Figure 4) interact with the SC portion of hpIgR, whereas a polypeptide containing residues 1-101 of CpbA did not bind to hpIgR.

Another bacterial protein, Streptococcus pneumoniae SpsA along with the aforementioned CbpA independently binds a region of human and mouse pIgR. Use of these proteins as binding regions of a polyspecific binding molecule would be advantageous for targeting polyspecific binding molecules to pIgR. Binding a non-secretory region of pIgR can explain how bacteria are able to use pIgR to gain access to the cellular interior because binding to the secretory component in bodily/humoral secretions would sequester the bacteria, and cell entry would be inefficient. SpsA and CbpA are Streptococcus virulence factors that bind to the human polymeric immunoglobulin receptor. The SpsA sequence YRNYPT binds to pIgR. CbpA has 2 RRNYPT sequences. This sequence is responsible for binding to pIgR, and inhibiting binding of CbpA to pIgR prevents bacterial invasion.

Small polypeptides that retain the ability to interact with human and animal species of pIgR are utilized as pIgR targeting elements in the fusion proteins of the invention. Such binding regions may include those identified by phage display of disulfide constrained peptides as described above or polypeptides including but are

not limited to the CbpA1, CbpA2, and CbpA3 polypeptides described by Zhang et al. In addition, other polypeptides from bacterial proteins homologous with CpbA, the pneumococcal adhesin protein in Streptococcus pneumoniae studied by Zhang et al., are of use as pIgR targeting elements and are therefore considered part of the present invention. Such homologous proteins are present in virtually all pneumococcal serotypes. Those skilled in the art will be able to identify additional homologous proteins from genomic and protein databases such as Swiss-Prot, Entrez, and GenBank.

A search of Swiss-Prot revealed the following list of proteins (listed by Accession Number) that have sequences homologous with R1 and R2: O30874, O69188, O33741, O33742, Q9RQT5, AAF73779, AAF73781, AAF73788, AAF73814, AAF73790, Q9RQT3, Q9RQT2, AAF73776, AAF73786, AAF73792, AAF73798, AAF73807, AAF73810, AAF73812, AAF73822, AAF73795, Q9RQT6, AAF73785, Q9ZAY5, Q9RQT4, Q9RQT1, AAF73777, AAF73799, AAF73801, AAF73809, AAF73784, AAF73817, AAF73778, AAF73811, AAF73813, O33753, AAF73787, AAF73808, AAF73773, AAF73780, AAF73797, AAF73775, AAF73791, AAF73804, AAF73816, BAB01952, O58288, Q9Y102, and Q54972.

Smaller polypeptides comprising portions of the entire sequence of CbpA and proteins homologous to CbpA, and preferably portions of R1 and R2 and polypeptides homologous to R1 and R1, are identified based on their ability to bind to animal species of pIgR, preferably human pIgR. An overlapping, nested set of peptides is synthesized and their ability to interact with pIgR can be tested to identify peptides that may be used as pIgR-targeting elements. The peptides may be tested for their ability (i) to prevent SC binding to pIgR coated beads or (ii) to prevent adherence, invasion, or transmigration by S. pneumoniae R6x to Detroit cells, both methods being described by Zhang et al. The polypeptides may be from 5 to 100 amino acids long, preferably from 6 to 50, and most preferably from 6 to 20. An offset of 1 to 5 amino acids and preferably 3 to 4 amino acids may be used. A nested, overlapping set of peptides 15 amino acids long with an offset of 3 amino acids that would contain residues 1-15, 4-18, 7-21, 10-24, 13-27, etc., until the last residue in the polypeptide sequence is reached. By comparing the amino acids in peptides that are contiguous in CpbA that show positive binding to pIgR, the core

linear sequence that is required for binding to pIgR is identified. A large peptide may be systematically reduced in size until the smallest peptide that produces a positive binding to pIgR is identified. Techniques for identifying the core linear sequence have been described by Geysen et al. (J. Immunol. Methods 102:259-274, 1987), Tribbick et al. (J. Immunol. Methods 139:155-166, 1991), Geysen et al. (J. Molecular Recognition 1:32-41, 1988), and Tainer et al. (Mol. Immunol. 23:709-715, 1986).

A polypeptide containing the smallest peptide that produces a positive binding to pIgR together with 0 to 15 amino acids, preferably 0 to 10, and most preferably 0 to 5 amino acids at the amino and/or carboxyl terminus of that sequence may be used as the pIgR binding region. The peptide is further modified by systematically identifying the most important residues in the core sequence by replacing each residue, one at a time, with alanine and quantitating the ability of the modified peptide to inhibit SC binding to pIgR. Amino acids in the core sequence are replaced with any of the known amino acids, and the resulting effects on SC binding to pIgR quantitated. By comparing the peptides for the quantitative ability to inhibit SC binding to pIgR, the most effective peptide sequences are identified. It is anticipated that sequences that are not identical to those in CpbA may be identified and used as binding regions. Further modifications to the peptides may be introduced by tethering the ends of the peptides by adding cysteine residues (1 to 2) at each end of the peptide so that a disulfide bonded loop containing the core sequences (or modifications thereof) are contained in the loop. Additionally or alternatively, non-contiguous amino acid sequences may be linked together using a short $(Gly_4\text{-}Ser)_x$ sequence, wherein x may be from 1 to 10, and used as pIgR binding regions.

## EXAMPLE 3: FUSION PROTEINS COMPRISING PIGR TARGETING SINGLE-CHAIN ANTIBODIES

### A. VIRAL ENVELOPE PROTEINS

This example describes strategies aimed at genetically engineering recombinant viral envelope proteins in which retroviral envelope gene (env) sequences are linked in frame to the pIgR ligand coding sequence to confer new binding specificity to the viral envelope. Techniques for altering the binding

specificities of envelope proteins are reviewed in Cosset et al., S. Gene Ther 3: 946-956, 1996; and Schnierle et al., Gene Ther 3: 1069-1073, 1996.

Retroviral envelope proteins are synthesized as precursor forms that are processed into proteolytic products, the surface peptide (SU) which binds a cellular receptor, and the transmembrane peptide [TM] which tethers the SU:receptor complex to the viral envelope. Fusion proteins comprising single-chain antibodies (sFv) and env sequences have been can be incorporated into viral particles and are capable of recognizing cognate receptors in that context (Russell et al. Nucleic Acids Res 21: 1081-1085, 1993). Re-definition of host range target specificity of MuLV-based vectors has been reported with envelope chimeras incorporating ligands or scFvs directed to the EGF receptor (Chen et al. FEBS Letters 338:167-169, 1994; Cosset et al., J Virol. 69:6314-6322, 1995), IGF-I receptor (Chadwick et al., J. Mol. Biol. 285:485-494, 1999), the LDL receptor (Somia et al. Proc. Natl. Acad. Sci. USA 92:7570-7574, 1995), the ErbB-2 receptor (Schnierle et al., Gene Ther 3:334-342, 1996) and the tumor-specific melanoma-associated antigen (Martin et al., Human Gene Therapy 9:737-746, 1998). Similarly, sFvs have been substituted for various portions of the Spleen Necrosis Virus (SNV) SU peptide as well as the N-terminus of the TM peptide (Chu et al., Gene Ther 1:292-299, 1994; Chu et al., R. J. Virol. 69:2659-2663, 1995; J. Virol. 71:720-725, 1997).

Fusion proteins comprising a pIgR-targeting element (e.g., sFv-5 or sFv-5AF) and the N-terminus of the SU peptide region of retroviral env genes (MoMLV, MLV, SNV, etc.), internal portions of the SU peptide, or the N-terminus of the TM peptide are prepared (Figure 12).

Env-sFv fusion proteins are engineered to include spacer or linker peptides between the pIgR ligand and the Env peptides to evaluate their effect on receptor binding and internalization. Spacers are designed to allow for more conformational flexibility, enhanced viral vector fusogenicity and increased viral transduction (Valesesia-Wittmann et al., J Virol. 70: 2059-2064, 1996; Lavillette et al., J Virol 72: 9955-9965, 1998). Spacers of linker peptides can be protease-activatable thereby allowing for two-step targeting (Nilson, BHK et al. Gene Ther 3: 280-286, 1996). Upon cell surface binding through the pIgR ligand-pIgR complex formation, the fusion Env-sFv protein is cleaved and internalization proceeds via the natural viral

entry pathway. In this modality, pIgR, the pIgR stalk molecule and/or the pIgR secretory component are proteins that may be displayed on a cellular surface that are targeted as displayed proteins rather than as transcytotic mediators, although the two activities are not mutually exclusive.

### B. VIRAL CAPSID DOMAINS

Adenovirus capsid is an icosohedral structure in which the faces are made up of hexon subunits and the vertices of penton bases. Each penton base interacts with an adenoviral fiber molecule, which has a globular domain called the knob. Genetically engineered chimeric adenoviral fiber knob domains can be re-targeted to receptors other than those used by wildtype virions. For pIgR-targeted virions, fusion proteins comprise a pIgR ligand and fiber sequences such as (a) an internal fiber sequence, exclusive of the penton binding region, thereby replacing the terminal fiber and knob domains with the pIgR ligand; (b) everything but the globular knob domain of the fiber protein, thereby replacing the knob domain with a pIgR ligand; and (c) the knob domain itself. See Figure 14.

### C. TRANSMEMBRANE PROTEINS AND/OR DOMAINS

Alphavirus vectors, such as the Sinbus virus, consist of an icosohedral nucleocapsid surrounded by a viral membrane (lipid bilayer). In the membrane are two virally encoded structural glycoproteins, E1 and E2, that form heterodimers with E2 mediating binding to the wildtype virus' natural receptor. Fusion E2 glycoproteins in which a cell-targeting ligand is fused to the E2 sequence have been described as facilitating targeted infection by the virion (Ohno, K et al. Nature Biotech 15: 763-767, 1997). Fusion glycoproteins comprising E2 sequences and sFv-5 are prepared and incorporated into alphaviral virions in order to generate pIgR-targeted membrane-bounded vesicles and virions.

## EXAMPLE 4: DIABODIES AND BI-SPECIFIC ANTIBODIES

A different strategy uses bispecific binding molecules (e.g., antibodies) to preferentially target gene delivery vectors to selected cell types (Figure 13). In brief, an antibody (or other binding molecule) that binds both the gene delivery vector and a protein (e.g., a receptor or surface antigen) preferentially displayed on the cells of interest. The bivalent binding molecule acts as a "bridge" between the cellular

surface molecule and the gene delivery vector. See, e.g., U.S. Patent No. 5,712,136. Multivalent, including bivalent, pIgR-targeted proteins are described in U.S. patent application Serial No. 60/267,601, attorney docket No. 057220.0401, entitled "Polyspecific Binding Molecules Having a Polymeric Immunoglobulin Receptor Binding Region" by Houston, L.L., and Sheridan, Philip L., filed February 9, 2001.

Such proteins direct membrane-bounded vesicles and virions to pIgR-displaying cells are bi-specific antibodies or, more generally, diabodies. Such fusion proteins have the capacity to bind to at least two different epitopes, one of which is a pIgR molecule, a pIgR stalk molecule, or a pIgR SC molecule, the other of which is found on the surface of a vesicle or virion. The diabody thus acts as a "bridge" that is associated with a pIgR molecule and a vesicle or virion. See Figures 13(e) and 16.

The non-pIgR epitope that is recognized by a diabody of the invention may be, by way of non-limiting example, the following viral proteins. For retroviruses, the epitope may be found at the N- or C-terminus of the Env SU peptide, internal domains or epitopes of the SU peptide, or the N-terminus of the Env TM peptide. For adenoviral vectors, the penton base structure, internal amino acid epitopes of the adenoviral fiber, terminal peptide sequences of the adenoviral fiber lacking the globular knob domain, and the fiber globular "knob" domain. For alpahviruses, the exposed terminus of the E2 glycoprotein, and any exposed epitope of the E2 glycoprotein (Figure 15). For AAV, epitopes contained on the VP3 capsid protein, epitopes contained on any of the 2 individual VP1 and VP2 capsid molecules, and any exposed epitope created due to the collective general conformation of the 3 (VP1, VP2, VP3) capsid proteins. For HSV, epitopes present on glycoprotein I and/or glycoprotein E.

## EXAMPLE 5: PREPARATION AND MANUFACTURING OF VIRIONS USING STABLE CELL LINES

### Choice of parental cell lines

Human kidney 293 cells (ATCC CRL 1573), human fibrosarcoma HT-1080 cells (ATCC CCL 121), canine sarcoma D-17 cells (ATCC CRL 8468) are several of the most common cell lines used for the generation of packaging and producer cell

lines. The 293 and HT-1080 cell lines were selected for the establishment of restroviral packaging cell lines because they provide good overall titers that lack endogenous murine retroviruses, the latter being a preferred safety feature. See, e.g., U.S. Patent 5,591,624; Rigg, R.J., Chen, J., Dando, J.S., Forestall, S.P., Plevac, I., and Bohnlein, E. A novel human amphotropic packaging cell line: high titer, complement resistance, and improved safety. Virology 218:290-195, 1996; Forestell, S. P., Dando, J. S., Chen, J., de Vries, P., Boehnlein, E., and Rigg, R. J. Novel retroviral packaging cell lines: complementary tropisms and improved vector production for efficient gene transfer. Gene Ther. 4:600-610, 1997; Rasheed, S., Gardner, M.B., and Chan, E. Amphotropic host range of naturally occurring wild mouse leukemia viruses. J. Virol. 19:13-18, 1976; Pensiero, M.N., Wysocki, C.A., Nader, K., and Kikuchi, G.E. Development of amphotropic murine retrovirus vectors resistant to inactivation by human serum. Hum. Gene Ther. 7:1095-1101, 1996.

Parent cells are maintained in DMEM (Irvine Scientific, CA) supplemented with 10% gamma-irradiated defined fetal bovine serum (FBS, Hyclone Laboratories Inc., UT), 20 mM HEPES (Irvine Scientific, CA), 1X non-essential amino acids and 1 mM sodium pyruvate. Parent cell lines used to generate clinical vector producing cell lines are banked and tested in accordance with FDA guidelines for origin (i.e. isoenzyme analysis and karyotyping), absence of expressed retroviral sequences and any pathogens such as mycoplasma, bacteria, fungus and viruses.

**Generation and analysis of packaging cell lines (PCLs)**

Techniques used to generate packaging cell lines for the generation of retroviral vectors are described by Sheridan et al., Mol. Ther, 2000. 2: 262-275 and references therein. Similar strategies are used to generate PCLs for other viral systems such as lentivirusus, adenoviruses, adeno-associated viruses, alpha-viruses, etc. In general, structural proteins , such as retroviral gag/pol and env expression plasmids (or tat and rev for lentiviruses, rep and cap genes for AAV, etc.) are sequentially introduced into cells by $CaPO_4$-mediated co-transfection with different selectable marker plasmids followed by the appropriate selection for 2 weeks. Selected Gag/pol intermediate pools are analyzed for p30 expression and subsequently dilution cloned into 96-well plates according to protocols described below.

Gag/pol intermediate clones are analyzed for p30 expression as well as titer potential. The p30 expression is detected in a Western blot using polyclonal goat anti-p30 antibodies followed by secondary HRP-labelled anti-goat antibodies and chemiluminescent detection using standard procedures. Titer potential of the Gag/pol clones is tested by either transfecting or transducing both the amphotropic env and a retroviral vector carrying a marker gene, typically beta-gal or $neo^R$, to generate producer pools. Supernatants from transient and stable producer pools are harvested, filtered (0.45 um) and the titer determined as described below. Clones with the highest titer potential are co-transfected with a retroviral env expression plasmid and a marker, transfected cells are selected and dilution cloned. PCL clones are analyzed for gp70 expression and titer potential. Envelope proteins are detected in a Western blot using polyclonal goat anti-gp70 antibodies (Quality Biotech, MD) following standard procedures as described above. The titer potential is tested by several rounds of transduction of retroviral vectors into PCLs at a high ratio of vector to PCL in order to test the limits of the packaging capacity.

**Generation and analysis of vector producing cell lines (VPCLs)**

Retroviral producer pools and clones are established using a process called the "high MOT approach." MOT, the multiplicity of transduction, is defined as the number of infectious viral particles used per PCL cell for the production of VPCL pools. In high MOT approaches, it is preferable to use a MOT $>20$. Typically, the PCL is seeded at 1 x $10^5$ cells/well in a 6-well plate one day prior to transduction with the titered VSV-G-pseudotyped vector (see below) of interest. Two hours before transduction, fresh media containing polybrene (8 ug/ml final) is added. The appropriate volumes of vector supernatants are then added to PCLs corresponding to MOTs of 0.1, 0.5, 5, 25 and so on. After 20-24 hours the vector supernatant is replaced with 2 ml of fresh media. The transduction procedure is repeated for a second day using the same volume of vector supernatant. Producer pools are grown to confluency and supernatants collected daily at about 24, 48 and 72 hours post-confluency to determine pool TOE and PCR titers. Selected pools are then dilution cloned into 96-well plates and resulting single cell clones analyzed in several rounds of titer determination. Resulting pools are selected and the beta-gal titers from

filtered pool supernatants determined in triplicates using the Galacto-Light assay described below.

Generally, at least 100 clones are tested each time after introduction of a retroviral component at the Gag/pol intermediate, PCL and VPCL stages. Once candidate PCL and VPCL clones for production of clinical material are identified, manufacturer's master and working cell banks are generated and tested for a variety of parameters including (i) growth characteristics, (ii) stability of retroviral components, (iii) titer over extended culture periods (up to 6 months) and (iv) the absence of RCR, mycoplasma, bacterial contamination and other adventitious agents including bovine virus, HBV, EBV, Parvovirus B-19, AAV, CMV, HIV I and II, and HTLV I and II. Testing for eco- and amphotropic RCR is also performed.

**Large scale vector production**

Cell banks are used for large scale characterization, vector processing and stability studies. A number of reduced homology producer clones are further characterized in extended large-scale production runs. The highest titer vector producing clones are expanded to either 10-layer hotels, cell cubes or bioreactors. Large scale cultures that allow for concurrent monitoring of VPCL growth characteristics and extended vector production for over 14 days for retroviral vectors has been described (Sheridan, et al., 2000). Following large scale production, virions are prepared using size-exclusion and other conventional chromatography procedures.

**Detection of replication-competent retrovirus (RCR)**

Various procedures are used to determine the presence or absence of RCR in the VPCL or the vector product, respectively. To test the VPCLs, cells are seeded into culture with an equal number of cells of the replication permissive cell line, Mus dunni. VPCLs are seeded into flasks at a small scale (1 x $10^7$ cells) or roller bottles at a large scale (1 x $10^8$ cells). Cells are co-cultured for several passages and finally harvested. Cell free culture supernatant is tested using a marker rescue or PG4S+L- assay (Printz, et al. Gene Ther. 2: 143-150, 1995). An RCR producing cell line generated by infection of Mus dunni cells with a hybrid murine leukemia virus (Miller and Buttimore, Mol. Cell. Biol. 6: 2895-2902, 1986) serves as a positive control for the cocultivation procedure. Naïve Mus dunni cells serves as the negative control.

**Production of VSV-G pseudotyped supernatant**

Large scale production of concentrated VSV-G (Vesicular Stomatitis Virus Glycoprotein) pseudotyped vector supernatant (G-supernatant) is performed essentially as outlined by Yee et al. (Methods in Cell Biology 43: 99-112, 1994) with some modifications. Briefly, 293 cells are plated into T225 flasks at 1 x $10^7$ cells/flask. Twelve to 24 hours later the cells are $CaPO_4$-transfected with the VSV-G coding plasmid pMLP-G and the respective retroviral vector using the ProFection kit (Promega Corp., WI). Following incubation with the DNA precipitate for 6-8 hours, the DNA suspension is removed and fresh media added. Twelve to 24 hours later the supernatant is collected and fresh media is applied. Four to five repeat collections are made and the G-supernatant is pooled, filtered (0.45 um) and concentrated by centrifugation at 9,000 x g and 8°C for 8-18 hours. Pellets are resuspended in a small volume of fresh media, aliquoted, frozen under liquid nitrogen, and stored at -70°C. This concentrated viral supernatant is then evaluated for titer by transfer of expression (TOE, see below) and PCR titer analysis before carrying out high MOT generation of producer pools and clones.

## EXAMPLE 6: PREPARATION AND MANUFACTURING OF VIRIONS USING TRANSIENT TRANSFECTION

In certain cases stable packaging cell lines are not readily available for large scale vector production, in which case vector material is typically prepared by transient transfection (double or triple transfection). Production of vector by transient transfection relies on the ability to introduce the various vector plasmids (structural gene plasmids for gag, pol, and env genes together with the retroviral plasmid) into the parental cell lines by calcium-phosphate transfection. This can be carried out at different scales using either multiple 10 cm plates, T225 cm flasks, or 10-layer hotels. The basic procedure is outlined below for 10-layer hotel capacity, and a worksheet is included for the adaptation to smaller 10 cm and 225 cm scale. These guidelines are for seeding of 293T cells and may need to be optimized for other cell lines.

**Seeding of Tissue Culture 10-Layer Trays (Cell Hotels)**

Listed are some general guidelines for the efficient plating and large scale growth of cultured cell lines in 10-layer cell trays ("hotels"). Two parameters that influence cell growth and maintenance is the actual gas exchange rate and cell density(over confluent 293T cells often lift off the trays in sheets). As mentioned, due to (1) poor air exchange through the air vents on a 10-layer cell hotel and (2) a less than optimal air volume:liquid volume ratio (optimal is 10:1), it is beneficial to buffer formulated media with 20-40 mM HEPES. In addition, it is best to order media without L-glutatmine, adding it during the formulation step to a final concentration of 2-6 mM (depending on cell type). This will minimize the levels of glutamic acid and ammonia that can accumulate as a result of the breakdown of L-glutamine during long term storage (which is accelerated upon the addition of serum).

**Media Formulation for growth of 293T cells**

DME media, with 4500 mg/L glucose (Irvine Scientific, cat. # 9031)

10% fetal calf serum ("defined", gamma-irradiated, 40 nm filtered; Irvine Scientific)

2 mM L-glutamine (final conc. added within 1 week of use; 200 mM stock, 292 mg/L; Irvine Scientific)

1 mM sodium pyruvate (100 mM stock; Irvine Scientific)

1X nonessential amino acids (100X for MEM stock; Irvine Scientific)

20 mM HEPES (1M stock, 238.3 g/L water; Irvine Scientific)

**Expansion of cells for seeding of cell hotels**

Timetable "A" for a single 10-layer hotel: (5 liter vector prep)

Day -7: (day 1 refers to day of actual transfection):

Expand 293T cell stocks to 3 x T225 flasks at a density of $4e^7$ cells/30 mls media/flask.

Day -5:

Expand 293T cell stocks to 1 x 2-layer hotel at a density of $4\text{-}5e^7$ cells in 200-220 mls media. (plate 2 x T225 flasks at a density of $4e^7$ cells/30 mls media/flask for back up stocks).

Day -3:

Expand 2-layer hotel cell stock to 1 x 10-layer hotel at a density of $12.5e^7$ cells/1 liter media/flask.

Day 1:

The cell hotel should be 90-95% confluent. Add 800 mls to 1 liter of fresh complete media before carrying out the transfection.

Timetable "B" for a twelve (12) 10-layer hotels: (for a 60 liter vector prep)

Day -10: (day 1 refers to day of actual transfection)

Expand 293T cell stocks to 4 x T225 flasks at a density of $4e^7$ cells/30 mls media/flask.

Day -7:

Expand 293T cell stocks to 1 x 2-layer hotel at a density of $4\text{-}5e^7$ cells in 200-220 mls media. (plate 3 x T225 flasks at a density of $4e^7$ cells/30 mls media/flask for back up stocks).

Day -5:

Expand 2 x 2-layer hotel cell stocks to 2 x 10-layer hotels at a density of $12.5e^7$ cells/1 liter media/hotel.

Day -3:

Expand 2 x 10-layer hotel cell stocks to 12 x 10-layer hotels at a density of $12.5e^7$ cells/1 liter media/hotel. In addition, plate 1 x 2-layer hotel at $2.5\text{-}3e^7$ cells/220 mls media/hotel for use in evaluating the overall cell growth and confluency before carrying out the transfection.

Day 1:

Use the 2-layer cell hotel to check for overall cell confluency, which should be 90-95% confluent. Add 800 mls to 1 liter of fresh complete media before carrying out the transfection.

**Basic Transfection Procedure**

Step 1: Prepare DNA/1M $CaCl_2$/H2O mix. Aliquot 2X HBS into appropriate size tubes/bottles.

Step 2: While slightly agitating (vortexing), mix the DNA/$CaCl_2$ solution with 2X HBS in a dropwise fashion. Place the transfection mix at room temperature for 20-30 minutes.

Step 3: Overlay the tranfection mix onto cells and place at 37°C/10% $CO_2$ for 6-8 hours (or overnight if desired).

Step 4: Replace the transfection mix with fresh media and collect vector supernatant at regular intervals, e.g., 24, 32, 40, 48 and 56 hours after the start of transfection.

**Vector Harvesting**

Depending on the type of downstream processing procedures to be used to concentrate and purify the vector, the use of phenol red-free media may be warranted. Phenol red-free DME (Irvine Scientific, cat. # 9041) can be substituted for the standard DME and the media formulated with L-glutatmine shortly before use (within 1-2 weeks) to minimize harmful effects of breakdown products, as described above.

## EXAMPLE 7: ASSAYS FOR EVALUATING VIRAL VECTOR TRANSFER

**Transfer of expression (TOE) assay and titer determinations**

This general titering assay utilizes HT-1080 (retroviruses) or 293 (adenoviruses, AAV and retroviruses) target cells seeded one day prior to transduction at 3 x $10^5$ cells per well in a 6-well plate (Corning Costar, NY). Eight ug/ml polybrene is added 2 hours before transduction with serial dilutions of vector supernatants. After 20-24 hours the supernatant is replaced with 1-2 ml of fresh media. Cells are allowed to grow for an additional 24-48 hours before supernatants or genomic DNA are assayed for the expressed gene product (Transfer of Expression or TOE titer) or the number of provector copies present (PCR titer), as described below.

**Transfer of expression / transduction protocol for the titering of adenovirus and AAV vectors**

AAV vectors are titered on 293 cells, typically in the presence of Ad5(E1A-)-helper virus. Cells are incubated from 1 to 5 days from the time of cell plating to functional analysis. Ad5-helper is typically added at an MOT of 0.7, and, as a control, the transduction process can be inhibited by heparin.

The assay requires the following materials: Ad5-helper virus (typically $1e^8$ CFU/ml titer); AAV vector (potential titer typically designated in genome particles/ml); Heparin (2.5 mg/ml stock concentration; 50 ug/ul working concentration); AAV Standard vector; and 293 cells (grown in DMEM w/10% FBS/P&S).

The general protocol is as follows.

1. Seed $2e^5$ 293 cells in 6-well plates in 2 mls of media approximately 36-48 hours prior to the day of transduction (293 cells often take some time to achieve log growth after cell splitting).

2. Prior to transduction, replace the media on cells with 900 uls of fresh media. Count one well to determine the approximate cell number/well and calculate the amount of Ad5-helper needed for an MOT of 0.7.

3. Add the determined amount of Ad5-helper to each well as per the experimental design (be sure to set up an extra Ad5-helper alone control for cell count determinations; see Control 1 and Step 5 below). This is also the time at which to add the Heparin for your inhibition of transduction controls. The following controls are included:

Control 1: Ad5-helper alone (it may be necessary to titer the Ad-helper in a pilot experiment to determined the optimal MOT for efficient AAV transduction without cytopathic effects on the 293 cells).

Control 2: Inclusion of Heparin for AAV inhibition to confirm proper infection mechanism and rule out pseudotransduction.

4. Add the AAV vector sample with a 100 ul volume (brings the final volume to 1 ml) and transduce for 18-24 hours at 37°C.

5. Eighteen to 24 hours after transduction, count one of the Ad5-helper alone control wells (see Step 3 above) and determine the average cell number per well; this will be needed for Step 6B below.

6. For the remaining cell samples, very gently wash the cells with 2 mls of 1X PBS, and either:

A. Add 150 uls of lysis buffer for analysis of cell lysates (ex. beta-gal assay), or

B. Harvest the cells for FACS analysis as follows:

Add 0.5 mls trypsin / 0.5 mls media and collect cells. Wash wells with 0.5 mls media and combine with collected cells.

Pellet cells, flick to resuspend, and wash with 3 mls 1X PBS.

Resuspend the cells in 0.5 – 1 ml 1X PBS / 1% FBS (final concentraion of $1e^6$ cells/ml) and place on ice until ready to analyze by FACS.

## EXAMPLE 8: ASSAYS FOR EVALUATING GENE DELIVERY

For assays of gene delivery, the gene contained within the gene delivery vectors of the invention is beta-galactosidase or other reporter genes such as luciferase, and green fluorescent protein (GFP) and its derivatives (EGFP, BFP, RFP, etc.). These will allow for visualization of cells that were actively transformed by the viral vectors through either direct cell staining, fluorescence-based flow cytometric methods (for EGFP, etc.), luminescence (in the case of luciferase) or immunohistochemical staining.

**Post-infection beta-galactosidase assay**

Assays of post infection beta-galactosidase activity proceed according to protocols such as the following. The first method is a biochemical staining procedure in which transduced target cells are stained 48-72 hours post-transduction using x-gal staining following a standard procedure. The second procedure is a chemiluminescent detection method utilizing the Galacto-light Plus Kit (Tropix, Inc., MA), in which transduced target cells are treated as per the manufacturer's instructions and luminescence is read using a Tropix TR717 microplate luminometer (PE Applied Biosystems, CA).

To verify that the beta-galactosidase activity observed in the transduced cells is due to expression following reverse transcription and not the result of pseudotransduction of beta-galactosidase activity present in the vector preparations, HT1080 cells are infected in the presence or absence of 3'-azido-3'deoxythymidine (AZT, a.k.a. zidovudin, GlaxoWellcome). AZT, which inhibits reverse transcription, will inhibit true gene delivery but will not impact alternate delivery events, e.g., the pseudotransduction of beta-qalatactosidase.

Cells are incubated with 50 uM AZT for 24 hr prior to infection, and fresh AZT is added at the time infection. On day 1, HT1080 cells to which a final concentration of 50 uM (micromolar) AZT has been added are seeded at 4E4 cells/well/24 well plate (scaled up accordingly for larger wells). As a control, HT1080 cells to which no AZT are also prepared. The cells are incubate for about 24 to 48 hours with an AZT concentration of 35 mM. On day 2, a dilution series of vector particles (e.g. undiluted to 1/10,000 dilution) is prepared in medium containing 50 uM (micromolar) AZT (and polybrene if desired). Media is aspirated from HT1080 cells, and 350 ul of each vector particle dilution sample is added to individual wells. Control wells contain vector particles but no AZT; additional controls that contain no vector particles or AZT are used to examine and, if need be, correct for background staining. The cells are incubate for about 24 to 48 hours. Medium containing vector particles from transduced cells is aspirated, and the cells are washed several times with PBS. Cells are fixed and stained for reporter gene expression.

**Determination of titer by PCR**

Triplicate wells of target cells (HT-1080 or 293 cells for retroviral vectors) are transduced with serial dilutions of vector preparations in the presence of 8 ug/ml polybrene. 24 hours after transduction, fresh media is applied and cells grown for an additional 24-48 hours. Cells are washed with PBS, incubated with 2.5 ml of lysis buffer (100 mM Tris, pH 8, 5 mM EDTA, 0.2% SDS, 100 mM NaCl, 100 g/ml proteinase K) at 37 C for 2 hr, and the DNA then precipitated. Genomic DNA pellets are washed with 70% ethanol, resuspended in 500 1 TE buffer, and quantitated by staining with Hoechst dye H33258 (Sigma, MO) and compared directly against calf thymus DNA standards using the CytoFluor II fluorometer (PerSeptive Biosystems, MA). DNA samples are subjected to automated PCR employing a PE ABI Prism 7700 system (Perkin-Elmer Corp., CT) and a synthetic oligonucleotide primer sets directed against retroviral packaging signal sequences to yield an ~80-bp product. The resulting fluorescence is detected and provector copy number titer expressed as transduction units/ml (TU/ml). Transduction units are defined as the provector copy number per genome equivalent relative to a known copy number standard, and represent a true reflection of vector integration units.

## EXAMPLE 9: ASSAYS FOR EVALUATING TRANSEPITHELIAL GENE DELIVERY

A pIgR transcytosis assay that makes use of polarized MDCK cell lines (control cells), or MDCK cells that have been engineered to express a pIgR molecule, is used to evaluate transepithelial transport, including but not limited to forward (basolateral to apical) transcytosis, reverse (apical to basolateral) transcytosis, or paracellular transport, i.e, transport through gap junctions found between epithelial cells. More generally, the disclosure provides methods and compositions for evaluating vesicles or virions that undergo any type of active or passive transport across an epithelial cell or through an epithelial barrier.

MDCK cells are grown on porous transwell membranes. The porous membrane separates the two cellular compartments, the basolateral compartment and the apical compartment. The basolateral compartment is the membrane side on which the cells rest. The apical side of the MDCK cell layer does not contact the porous membrane. The MDCK cells, or other appropriate cells known to those skilled in the art, form a continuous and contiguous layer that is essentially impermeable to large molecules unless a specific transport system exists for those substances.

MDCK cells are transfected with various homologs, isoforms and derivatives (e.g., fusion proteins) of pIgR so that the pIgR is expressed and displayed on the surface of the cells. Expression vectors comprising any homologs of pIgR may be transfected into MDCK cells. Preferred pIgR homolgs include rat, mouse, monkey, rabbit, simian and human pIgR. Chinese Hamster Ovary (CHO) cells transfected with expression vectors for mouse pIgR are described by Asano et al. (J. Immunol. Methods 214, 131-139, 1998); and transgenic mice that overexpress murine pIgR are described by de Groot et al. (Transgenic Res. 8, 125-135,1999).

Other epithelial cells may be grown on transwell membranes and used to assay the transcytosis or paracellular transport of vesicles or virions. Such cells may or may not express pIgR. In cells where pIgR is not present, another type of transcytocic or paracellular transporting element or mechanism causes, promotes, enhances or mediates trancytosis or paracellular transport of pIgR-targeted vesicles or virions.

To evaluate reverse transcytosis, vesicles or virions are placed in the apical compartment and isolated in the basolateral compartment. Vesicles and virions that penetrate the cell layer are found in the basolateral compartment as opposed to the apical compartment into which they were initially introduced. Paracellular transport is assayed in essentially the same manner, although chelator-mediated disruption of gap junctions is used in some controls. To evaluate forward transcytosis, vesicles or virions are placed in the basolateral compartment and media from the apical compartment is assayed.

## EXAMPLE 10: TREATMENT OF CYSTIC FIBROSIS

Cystic fibrosis (CF) is a common mongenic inherited disorder with a high morbidity and mortality as a result of loss of the $Cl^-$ transporter receptor. The fact that heterozygotes are phenotypically normal suggests that introduction of a single CFTR gene should restore $Cl^-$ transport. Potentially, genetic correction of CF may require transformation of only about 10 to about 20% of the airway epithelia, and CF is the focus of several gene therapy trials (reviewed in Knoell et al., Am J. Health Syst. Pharm 55: 899-904, 1998). In vitro gene transfer studies using retroviruses, adenoviruses, and cationic lipids (Drumm et al., Cell 62: 1227-1223, 1990; Rich et al. Nature 347: 358-363, 1990; Olsen et al., Hum Gene Ther 3: 253-266, 1992; Zabner et al., Hum Gene Ther 5: 585-593, 1994; Egan et al., Nature 358: 581-584, 1992; Lee et al., Hum Gene Ther 7: 1701-1717, 1996) to deliver copies of the CFTR gene have demonstrated restoration of $Cl^-$ transport. However, preclinical studies of nonhuman primates revealed inefficient airway gene transfer by adenoviruses (Engelhardt et al, Hum Gene Ther 4: 759-769, 1993), and some have made similar observations regarding the delivery of genetic vectors using liposomes, AAV, and retroviruses (reviewed by Johnson, LG and Boucher, RC. Chapter 23, Gene Therapy: Therapeutic Mechanisms and Strategies, pp. 473-487, 2000).

For adenoviruses, it is believed that the lack of expression of the high affinity CAR receptor on the apical surface of well-differentiated airway epithelial cells (Zabner et al. J Clin Invest 100: 1144-1149, 1997; Pickles et al., J Virol 72: 6014-6023, 1998), is the barrier which results in decreased binding and a low rate of

adenoviral vector internalization. Interestingly, is has also been demonstrated integrins believed to facilitate uptake of adenoviral vectors (Wickham et al., Cell 73: 309-319, 1993) are also expressed at low levels in the apical membrane. Similarly, membrane-associated heparan sulfate proteoglycan, the receptor for AAV (AAV-2, the most common serotype used in AAV vectors, Summerford et al., J Virol 72: 1438-1445, 1998), has also been shown to be localized to the basal surface and not the apical surface in well-differentiated airway epithelial cells (Duan et al., Hum Gene Ther 9: 2761-2776, 1998). Additional barriers to transduction by AAV in undifferentiated cells is the persistence of AAV genomes as single-stranded episomes, which are not efficiently converted to double-stranded DNA, as is preferred for high level transgene expression (Ferrari et al., Virol 70: 3227-3234, 1996; Teramoto et al., J Virol 72: 8904-8912, 1998).

Resistance of airway epithelium to transformation by cationic liposomes has also been shown to result from two different mechanisms. For undifferentiated celllines, nuclear entry had been identified as the rate-limiting factor (Zabner et al., J. Biol. Chem 270: 18997-19007, 1995), whereas failure of DNA-liposome complex entry is limited in well-differentiated airway epithelial cells (Matsui et al., J. Biol. Chem. 272: 1117-1126, 1997).

For retroviral vectors, which can only efficiently transform dividing cells, lack of cell proliferation in well-differentiated airway epithelia serves as the major barrier (Olsen, Gene Ther 5: 1481-1489, 1998). The continued development of lentiviral vectors, which can transduce non-dividing cells may overcome the requirement for cell proliferation. Pseudotyping and fusion re-targeting strategies should also contribute to the preferential and enhanced ability to infect polarized cells where receptors are concentrated on the basolateral surface (Fuller et al., Cell 38: 65-77, 1984).

It is believed that vesicles and virions that target different pIgR molecules, which are present on the apical and/or basolateral surfaces of polarized airway epithelium, will allow for cellular binding and internalization via nasal delivery of the viral systems described above. Additionally or alternatively, it is believed that pIgR-targeted delivery systems will increase the overall effective dose of the delivery

vehicle and minimize the exposure to proteases present in CF sputum (Kitson et al., Gene Ther 6: 534-546, 1999).

## EXAMPLE 11: ASSAYS FOR GASTROINTESTINAL DELIVERY OF PIGR-TARGETED VESICLES AND VIRIONS

In order to determine the delivery of pIgR targeted membrane-bounded vesicles or virions from the gastrointestinal tract, the following procedures are used. A cannula is implanted into the jugular vein of a rat for the purpose of collecting blood samples at various times. Another cannula is implanted into a region of the intestine, jejunum, ileum, or colon, for the purpose of administering the therapeutic entity to the intestine. A 350-375 gram Spraque-Dawley rat is suitable for this purpose although other strains of rats may be used. The cannulae are guided under the skin so that they exit the skin directly between the shoulders of the rat. This position prevents the rat from damaging the cannula. A single rat per cage is required. The membrane-bounded vesicles or virions are administered to the rat 2 to 7 days after the cannulae are implanted. During this time, the rat is observed for its general health and to determine the patency of the cannulae.

Vesicles or virions of the invention are given to the rat through the intestinal cannula. Before administration, a sample of blood (approximately 200 microliters) is withdrawn through the jugular vein cannula. Samples of blood are collected at more-or-less regular intervals over an 8 to 48 hour period after administration. The jugular cannula is kept patent by using saline with a small amount of heparin to prevent clotting. The blood is collected into a 1.5 ml Eppendorf tube that contains 5 microliters of heparin to prevent clotting. The blood is kept on ice for up to about 1 hour, before it is centrifuged in a table top Eppendorf centrifuge for 30 to 60 seconds. The supernatant (plasma) is collected and stored in a suitable manner, usually by freezing at -80°C.

The plasma or other biological sample is assayed in various ways that reflect various steps in the delivery of therapeutic agents in vesicles or virions. Commercially available antibodies to viral envelope and capsid proteins are used to monitor the transport of virions from one location to another. In order to monitor the delivery and expression of a nucleic acid, a nucleic acid that encodes a detectable

polypeptide is used (a "reporter gene"). Non-limiting examples of detectable polypeptides that can be used as reporter genes include green fluorescent protein (GFP) and variants thereof, beta-galactosidase, luciferase, avidin and streptavidin, enzymes such as horse radish peroxidase, and the like. Ultimately, an expression element that encodes a therapeutic protein of interest is used and the protein is detected using ligands specific for that protein. For example, if the therapeutic protein of interest in human growth hormone (hGH), commercially available antibodies directed to hGH are used.

Immunoassays are used to detect antibodies such as those described above. An immunoassay such as, for example, an enzyme linked immunosorbent assay (ELISA) is used to determine the concentration of the protein. In this case, the protein is not radiolabelled; rather, an antibody that recognizes an epitope present in the protein is coated to the bottom of 96-well plates. After washing, the presence and quantity of the protein is determined by reacting it with a second antibody that is conjugated to a detectable enzyme such as, e.g., horse radish peroxidase or alkaline phosphatase. After washing, a substrate for horse radish peroxidase or alkaline phosphatase is incubated in the well. The substrate is itself detectable or yields a detectable product. The amount of the product is determined by spectrophotometry at an appropriate wavelength. A control curve (using known quantities of purified protein) is used to determine the concentration of the fusion protein in the plasma samples.

Similar experiments are conducted to examine the potential of pIgR-targeted vesicles or virions as components of compositions intended for rectal delivery, e.g., a suppository. In these experiments, the vesicles or virions is administered by a rectal tube. A catheter is inserted through the anus of an anesthetized rat. The urinary catheter inserted 7.5 cm through the anus allows for delivery of formulations within the colon.

## EXAMPLE 12: IN VIVO TESTING OF LUNG AEROSOL FORMULATIONS

This example describes procedures can be used to examine pIgR-targeted vesicles or virions for their capacity for administration via inhalation. In these

experiments, vesicles or virions of the invention are administered as an aerosol or microparticulate formulation to the nasal or pulmonary cavity.

**In vivo evaluation of pIgR-targeted liposomes and gene therapy vectors**

Rat and rabbit in vivo studies looking at superficial and deep lung transformation by various viral vectors have been described (Wang et al., Am. J. Respir. Cell Mol. Biol. 22: 129-138, 2000). Typically, 6-10 mM EGTA is used to disrupt gap junctions between cells in the apical surface to allow for retroviruses and lentiviruses to travers the epithelial barrier and bind to basal membrane receptors, after which they are internalized.

Similar airway exposure experiments are carried out in animals with or without prior perfusion with $Ca^{++}$ chelators, such as EGTA, which modulate paracellular permeability.

Some receptors for viruses are located on the basolateral side of epithelial cells,is based membrane to infect apical side-EGTA disrupts gap junctions, so, if pIgR-mediated, EGTA not needed. In such instances, apical binding, endocytosis, apical to basolateral (reverse) transcytosis of virions allows them to travel to locations where they can interact with their receptors. In this manner, gastrointestinal uptake of pIgR-targeted gene delivery vectors can proceed without disrupting the gap junctions.

Different sizes of unique particles have been developed for inhalation of medicines. Particles that are too large for phagacytosis by macrophages have been made (see Ben-Jebria et al., Inhalation System for Pulmonary Aerosol Drug Delivery in Rodents Using Large Porous Particles, Aerosol Sci. and Technol. 21:421-433, 2000). These particles are porous with a large geometric diameter ($\sim 10$ um) and low mass density ($< 0.1$ g/cm$^3$). Experimental models have been developed using rats and guinea pigs to measure the efficacy of drug delivery using these particles

Both polymeric and nonpolymeric excepients may be used to prepare these large particles with low mass density. The polymeric microsphere preparation is made of biodegradable poly(lactic acid-co-glycolic acid, a.k.a. PLGA) polymer particles. The nonpolymeric large porous particles consists of human serum albumin, lactose, dipalmitoyl phosphatidylcholine (DPPC) and albuterol sulfate. Other particles may also be prepared for the purpose of delivering substances to the lung.

Aerosols are made with a Spinhaler dry powder inhaler and characterized in vitro with a cascade impactor and Aerosizer as detailed in Ben-Jebria et al., 2000. Chambers in which aerosols may be studied are used for administration of substances. Aerosol doses are administered to one or several animal chambers at the same time. Animals are conscious under these conditions. Using a plethysmograph, the animal is aerosolized and measured in the same chamber. The entire configuration is intended not to interfere with the monitoring of respiratory parameters, even during the aerosolization period. Immediate responses can be observed, as well as the onset of longer responses which may take place before the aerosolization is complete. Up to 12 mouse plethysmographs may be aerosolized at once, or 4 rat or 4 guinea pig chambers. Pulmonary measurements continue throughout the process, i.e., before challenge, during challenge, and after challenge.

The nebulized mixture may be dry as this not only minimizes deposition of aerosolized material in the reservoir, in the animal chamber, and in the tubing leading to the animal chamber, but it also assures a smaller particle size arriving at the animal. Obligate nose breathers, such as rodents, require a small particle size to allow penetration of the aerosol beyond the head passageways.

In another protocol, an animal, such as a rat or guinea pig, is anaesthetized and placed supine on a surgical board. The proximal trachea is exposed and a blunt cannula inserted and connected to a ventilator. The animal is still able to breathe through proximal and upper airways because the outer diameter of the cannula is 1 mm whereas the inner diameter of the trachea is 3-4 mm. (The 1 mm diameter cannula has greater airway resistance than the 3-4 mm airway). A pipette with a rubber bulb is attached to the inhalation side of the ventilator tubing for the purpose of introducing the particles. The bulb is filled with a set amount of particles and a portion is introduced as the operator squeezes it while the respirator is set to inhale. The portion is delivered in one 30 to 60 sec insufflation by forced ventilation at 3 ml tidal volume and 100 strokes/min frequency. Several repetitions of the procedure deliver 3 to 5 mg of particles over a period of 10-15 min.

The bronchoalveolar lavage fluid (BALF) is collected 10 min after the inhalation procedure and analyzed for the presence or absence of gene delivery

vectors, the nucleic acids transported thereby, and/or proteins expressed therefrom. Protocols for these types of assays are described in the preceding Examples.

The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising," "including," containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

The contents of the articles, patents, and patent applications, and all other documents and electronically available information mentioned or cited herein, are hereby incorporated by reference in their entirety to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. Applicants reserve the right to physically incorporate into this application any and all materials and information from any such articles, patents, patent applications, or other documents.

## CLAIMS

1. A fusion protein comprising a transepithelial delivery element from a first protein and a transmembrane domain from a second protein.

2. The fusion protein of claim 1, further comprising a polypeptide selected from the group consisting of a detectable polypeptide, a protein purification element, a cell-specific targeting element, a spacer, and a nuclear localization element.

3. A fusion protein comprising a transmembrane domain and a targeting element directed to a protein selected from the group consisting of a pIgR molecule, a pIgR stalk molcule, and a pIgR secretory component molecule.

4. The fusion protein of claim 3, wherein said pIgR is selected from the group consisting of a simian pIgR, a murine pIgR, a rodent pIgR, a rabbit pIgR, a bovine pIgR, or a human pIgR.

5. The fusion protein of claim 1, wherein said fusion protein confers the ability to undergo a process selected from the group consisting of apical endocytosis, basolateral endocytosis, apical exocytosis, basolateral exocytosis, apical to basolateral transcytosis, and basolateral to apical transcytosis.

6. A membrane-bounded vesicle comprising a fusion protein that comprises a transepithelial delivery element from a first protein and a transmembrane domain from a second protein, wherein the epithelial delivery element is displayed on the exterior of said membrane-bounded vesicle.

7. The membrane-bounded vesicle of claim 6, wherein said membrane-bounded vesicle is a liposome.

8. The liposome of claim 7, wherein said vesicle contains one or more biologically active molecules.

9. The liposome of claim 8, wherein said biologically active molecule is selected from the group consisting of a nucleic acid, a protein and a small molecule.

10. The liposome of claim 9, wherein said biologically active molecule is selected from the group consisting of a nucleic acid, a protein and a small molecule.

11. The liposome of claim 9, wherein said biologically active molecule is a therapeutic nucleic acid.

12. The liposome of claim 11, wherein said therapeutic nucleic acid contains an expression element that encodes a biologically active polypeptide.

13. A pharmaceutical composition comprising the liposome of any of claims 7 to 12.

14. A method of treating a disease or disorder comprising administering the pharmaceutical composition of claim 13 to an animal in need thereof.

15. A method of gene therapy comprising delivering a therapeutic nucleic acid to an animal in need thereof, wherein said delivering is via the liposome of claim 11.

16. The membrane-bounded vesicle of claim 6, wherein said membrane-bounded vesicle is a virion.

17. The virion of claim 16, wherein said virion contains a biologically active nucleic acid.

18. The virion of claim 17, wherein said biologically active nucleic acid is a therapeutic nucleic acid.

19. The virion of claim 18, wherein said therapeutic nucleic acid contains an expression element that encodes a biologically active polypeptide.

20. A pharmaceutical composition comprising the virion of any of claims 16 to 19.

21. A method of gene therapy comprising delivering a therapeutic nucleic acid to an animal in need thereof, wherein said delivering is via the virion of claim 18.

22. A fusion protein comprising a transepithelial delivery element from a first protein and a viral sequence that confers the ability to be associated with or incorporated into an envelope protein of a virus.

23. The fusion protein of claim 22, wherein said envelope protein is derived from a virus selected from the group consisting of gibbon ape Leukemia virus (GaLV), HTLV-I, MuLV, FIV and HIV.

24. The fusion protein of claim 22, wherein the amino acid sequence of said transepithelial delivery element replaces a receptor-targeting amino acid sequence of an envelope protein of a retrovirus.

25. The fusion protein of claim 22, wherein said envelope protein can be incorporated into a pseudotyped virion.

26. A virion comprising a fusion protein comprising a transepithelial delivery element from a first protein and a viral sequence that confers the ability to be associated with or incorporated into an envelope protein of a virus, wherein said transepithelial delivery element is displayed on the surface of said virion.

27. The virion of claim 26, wherein said virion contains a biologically active nucleic acid.

28. The virion of claim 27, wherein said biologically active nucleic acid is a therapeutic nucleic acid.

29. The virion of claim 28, wherein said therapeutic nucleic acid contains an expression element that encodes a biologically active polypeptide.

30. A pharmaceutical composition comprising the virion of any of claims 26 to 29.

31. A method of gene therapy comprising delivering a therapeutic nucleic acid to an animal in need thereof, wherein said delivering is via the virion of claim 28.

32. A fusion protein comprising a transepithelial delivery element from a first protein and a viral sequence that confers the ability to be associated with or incorporated into a capsid protein of a virus.

33. A nucleocapsid comprising a fusion protein comprising a transepithelial delivery element from a first protein and a viral sequence that confers the ability to be associated with or incorporated into a capsid protein of a virus, wherein said transepithelial delivery element is displayed on the surface of said nucleocapsid.

34. The nucleocapsid of claim 33, wherein said nucleocapsid contains a biologically active nucleic acid.

35. The nucleocapsid of claim 34, wherein said biologically active nucleic acid is a therapeutic nucleic acid.

36. The nucleocapsid of claim 35, wherein said therapeutic nucleic acid contains an expression element that encodes a biologically active polypeptide.

37. A pharmaceutical composition comprising the nucleocapsid of any of claims 33 to 36.

38. A method of gene therapy comprising delivering a therapeutic nucleic acid to an animal in need thereof, wherein said delivering is via the nucleocapsid of claim 33.

39. A packaging cell line that produces the virion of claim 15 or 26 or the nucleocapsid of claim 33.

40. A method for the ex vivo transformation of cells comprising contacting the virion of claim 15 or 26 or the nucleocapsid of claim 33 with cells that have been temporarily removed from an animal.

41. The virion of claim 12 or 29 or the nucleocapsid of claim 36, wherein said biologically active polypeptide is one that enhances the production, level or activity of a separately added biologically active molecule.

42. The virion or nucleocapsid of claim 41, wherein said biologically active polypeptide is an enzyme that catalyzes the conversion of a nucleobase, nucleoside or nucleotide analog into a nucleoside triphosphate analog.

43. A pharmaceutical composition comprising the virion or nucleocapsid of claim 41.

44. A method of treating an animal suffering from a disease or disorder, comprising contacting said animal with (a) the virion or nucleocapsid of claim 41 and (b) a biologically active molecule that is a substrate for a reaction or process mediated by said biologically active protein.

45. A method of treating an animal suffering from a disease or disorder, comprising contacting said animal with (a) the virion or nucleocapsid of claim 42 and (b) a nucleobase, nucleoside or nucleotide analog.

46. The method of claim 45, wherein said nucleobase, nucleoside or nucleotide analog is selected from the group consisting of AZT, ddI, ddC, ganciclovir (GCV), acylovir (ACV), dOTC, DAPD, PMPA, FddA, Abacavir (ABC), Epvir®, Hivid®, Retrovir®, Videx®, Zerit®, Zalcitabine (ddC), Stavudine (d4T), Lamivudine (3TC), .

47. The method of claim 46, wherein said biologically active protein is a thymidine kinase.

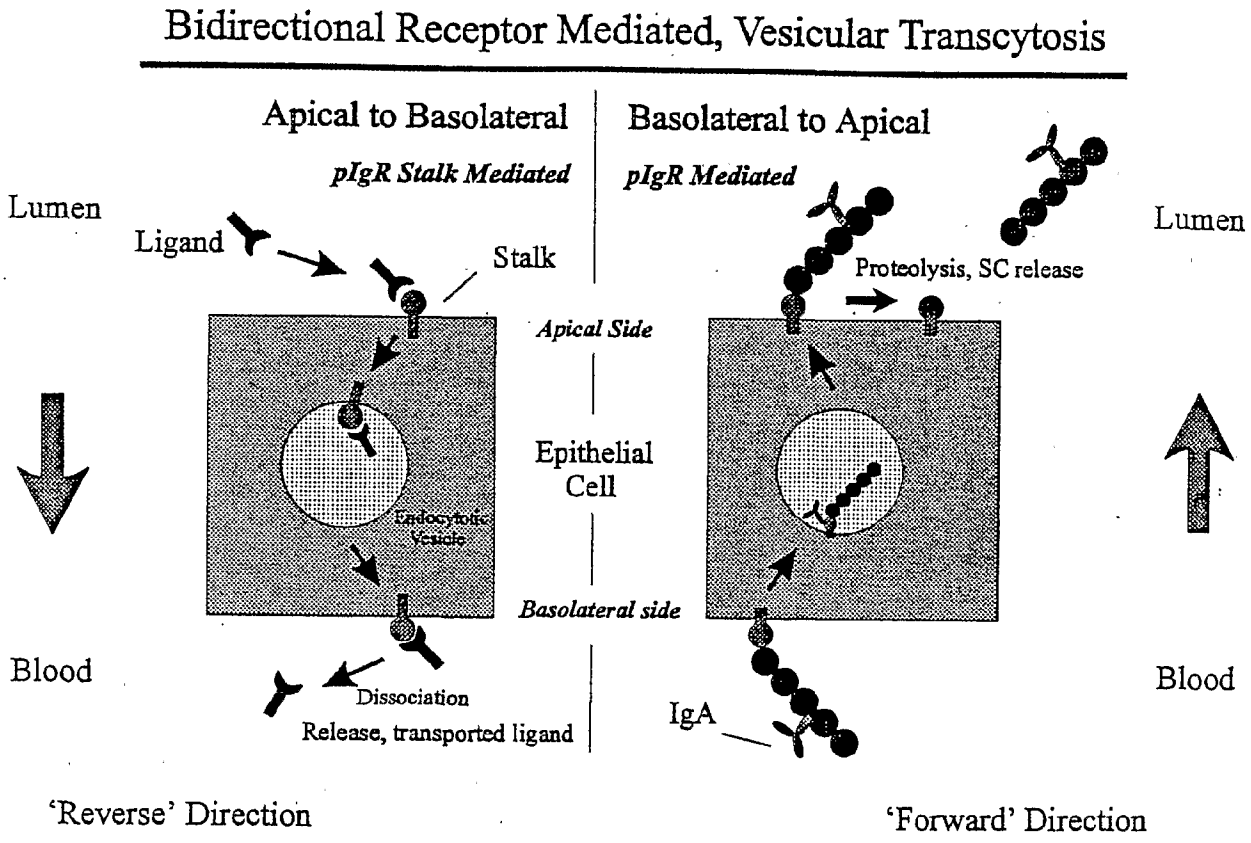

Bidirectional Receptor Mediated, Vesicular Transcytosis
Apical to Basolateral
pIgR Stalk Mediated
Basolateral to Apical
pIgR Mediated
Lumen
Ligand
Stalk
Proteolysis, SC release
Lumen
Apical Side
Epithelial Cell
Endocytotic Vesicle
Basolateral side
Blood
Dissociation
Release, transported ligand
IgA
Blood
'Reverse' Direction
'Forward' Direction

FIGURE 1

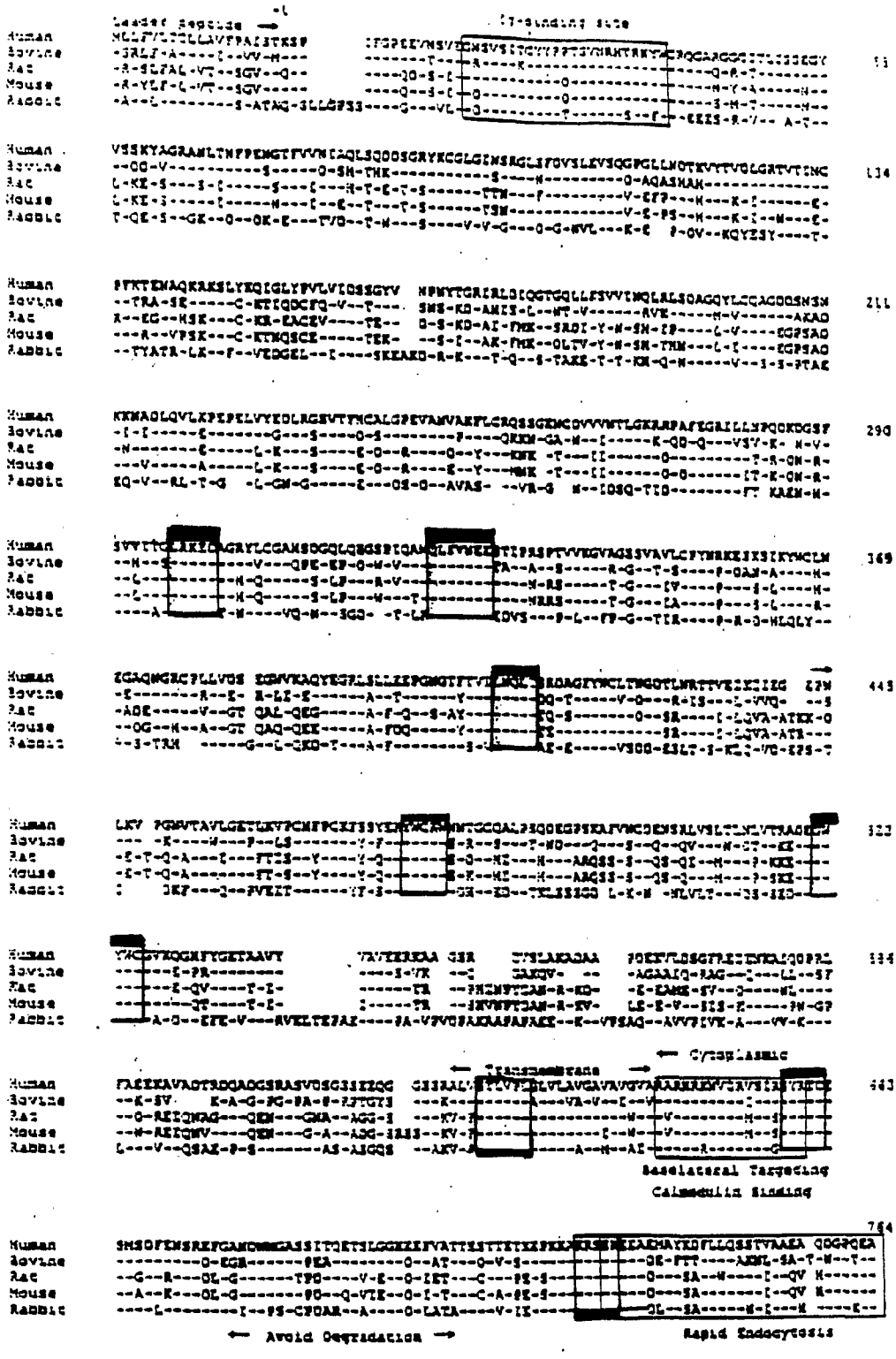

Human
Bovine
Rat
Mouse
Rabbit
Avoid Degradation
Rapid Endocytosis
Basolateral Targeting
Calmodulin Binding
Cytoplasmic

FIGURE 2A

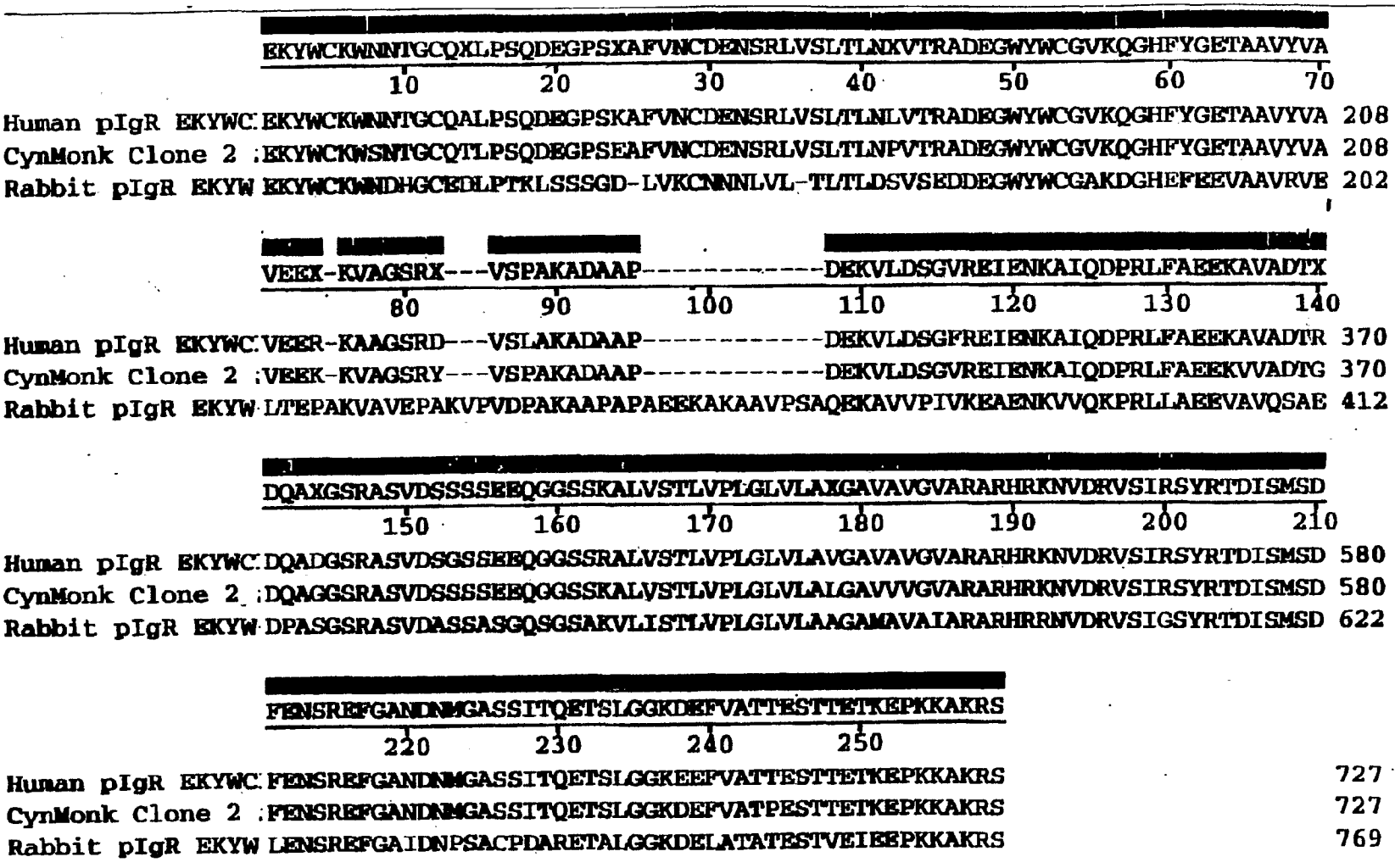

EKYWCKWNNTGCQXLPSQDEGPSXAFVNCDENSRLVSLTLNXVTRADEGWYWCGVKQGHFYGETAAVYVA
10 20 30 40 50 60 70
Human pIgR EKYWC EKYWCKWNNTGCQALPSQDEGPSKAFVNCDENSRLVSLTLNLVTRADEGWYWCGVKQGHFYGETAAVYVA 208
CynMonk Clone 2 EKYWCKWSNTGCQTLPSQDEGPSEAFVNCDENSRLVSLTLNPVTRADEGWYWCGVKQGHFYGETAAVYVA 208
Rabbit pIgR EKYW EKYWCKWNDHGCEDLPTKLSSSGD-LVKCNNNLVL-TLTLDSVSEDDEGWYWCGAKDGHEFEEVAAVRVE 202
VEEX-KVAGSRX---VSPAKADAAP----------DEKVLDSGVREIENKAIQDPRLFAEEKAVADTX
80 90 100 110 120 130 140
Human pIgR EKYWC VEER-KAAGSRD---VSLAKADAAP----------DEKVLDSGFREIENKAIQDPRLFAEEKAVADTR 370
CynMonk Clone 2 VEEK-KVAGSRY---VSPAKADAAP----------DEKVLDSGVREIENKAIQDPRLFAEEKVVADTG 370
Rabbit pIgR EKYW LTEPAKVAVEPAKVPVDPAKAAPAPAEEKAKAAVPSAQEKAVVPIVKEAENKVVQKPRLLAEEVAVQSAE 412
DQAXGSRASVDSSSSEEQGGSSKALVSTLVPLGLVLAXGAVAVGVARARHRKNVDRVSIRSYRTDISMSD
150 160 170 180 190 200 210
Human pIgR EKYWC DQADGSRASVDSGSSEEQGGSSRALVSTLVPLGLVLAVGAVAVGVARARHRKNVDRVSIRSYRTDISMSD 580
CynMonk Clone 2 DQAGGSRASVDSSSSEEQGGSSKALVSTLVPLGLVLALGAVVVGVARARHRKNVDRVSIRSYRTDISMSD 580
Rabbit pIgR EKYW DPASGSRASVDASSASGQSGSAKVLISTLVPLGLVLAAGAMAVAIARARHRRNVDRVSIGSYRTDISMSD 622
FENSREFGANDNMGASSITQETSLGGKDEFVATTESTTETKEPKKAKRS
220 230 240 250
Human pIgR EKYWC FENSREFGANDNMGASSITQETSLGGKEEFVATTESTTETKEPKKAKRS 727
CynMonk Clone 2 FENSREFGANDNMGASSITQETSLGGKDEFVATPESTTETKEPKKAKRS 727
Rabbit pIgR EKYW LENSREFGAIDNPSACPDARETALGGKDELATATESTVEIEEPKKAKRS 769

FIGURE 2B

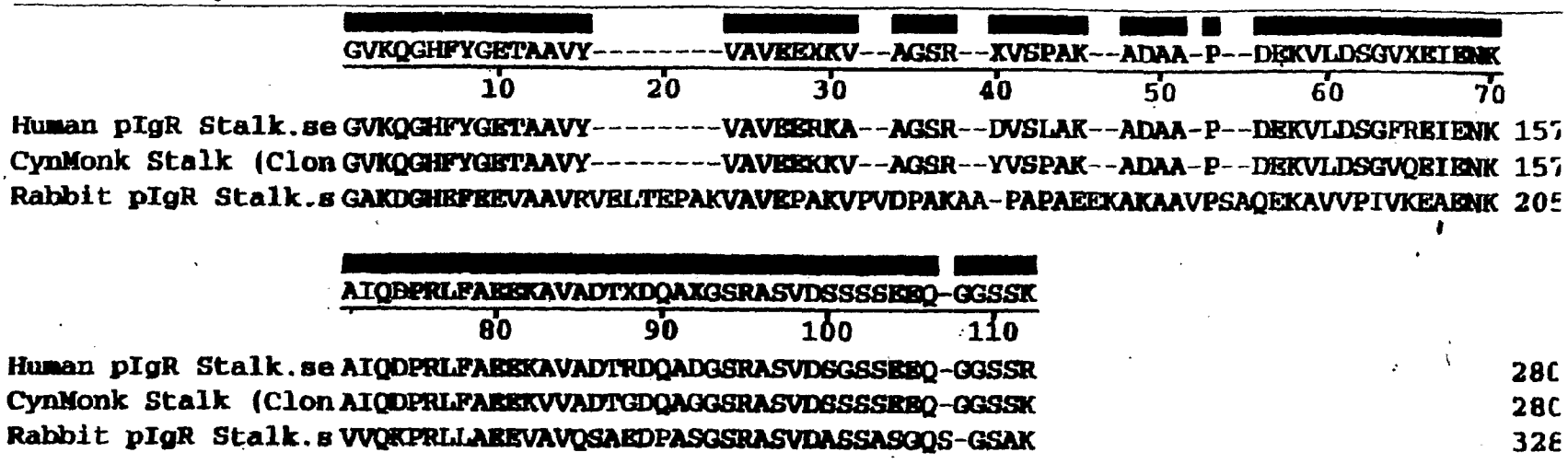

GVKQGHFYGETAAVY--------VAVEEKKV--AGSR--XVSPAK--ADAA-P--DEKVLDSGVXEIENK
10 20 30 40 50 60 70
Human pIgR Stalk.se GVKQGHFYGETAAVY--------VAVEERKA--AGSR--DVSLAK--ADAA-P--DEKVLDSGFREIENK 157
CynMonk Stalk (Clon GVKQGHFYGETAAVY--------VAVEEKKV--AGSR--YVSPAK--ADAA-P--DEKVLDSGVQEIENK 157
Rabbit pIgR Stalk.s GAKDGHEPEEVAAVRVELTEPAKVAVEPAKVPVDPAKAA-PAPAEEKAKAAVPSAQEKAVVPIVKEAENK 205
AIQDPRLFAEEKAVADTKDQAXGSRASVDSSSSEEQ-GGSSK
80 90 100 110
Human pIgR Stalk.se AIQDPRLFAEEKAVADTRDQADGSRASVDSGSSEEQ-GGSSR 280
CynMonk Stalk (Clon AIQDPRLFAEEKVVADTGDQAGGSRASVDSSSSEEQ-GGSSK 280
Rabbit pIgR Stalk.s VVQKPRLLAEEVAVQSAEDPASGSRASVDASSASGQS-GSAK 328

FIGURE 2C

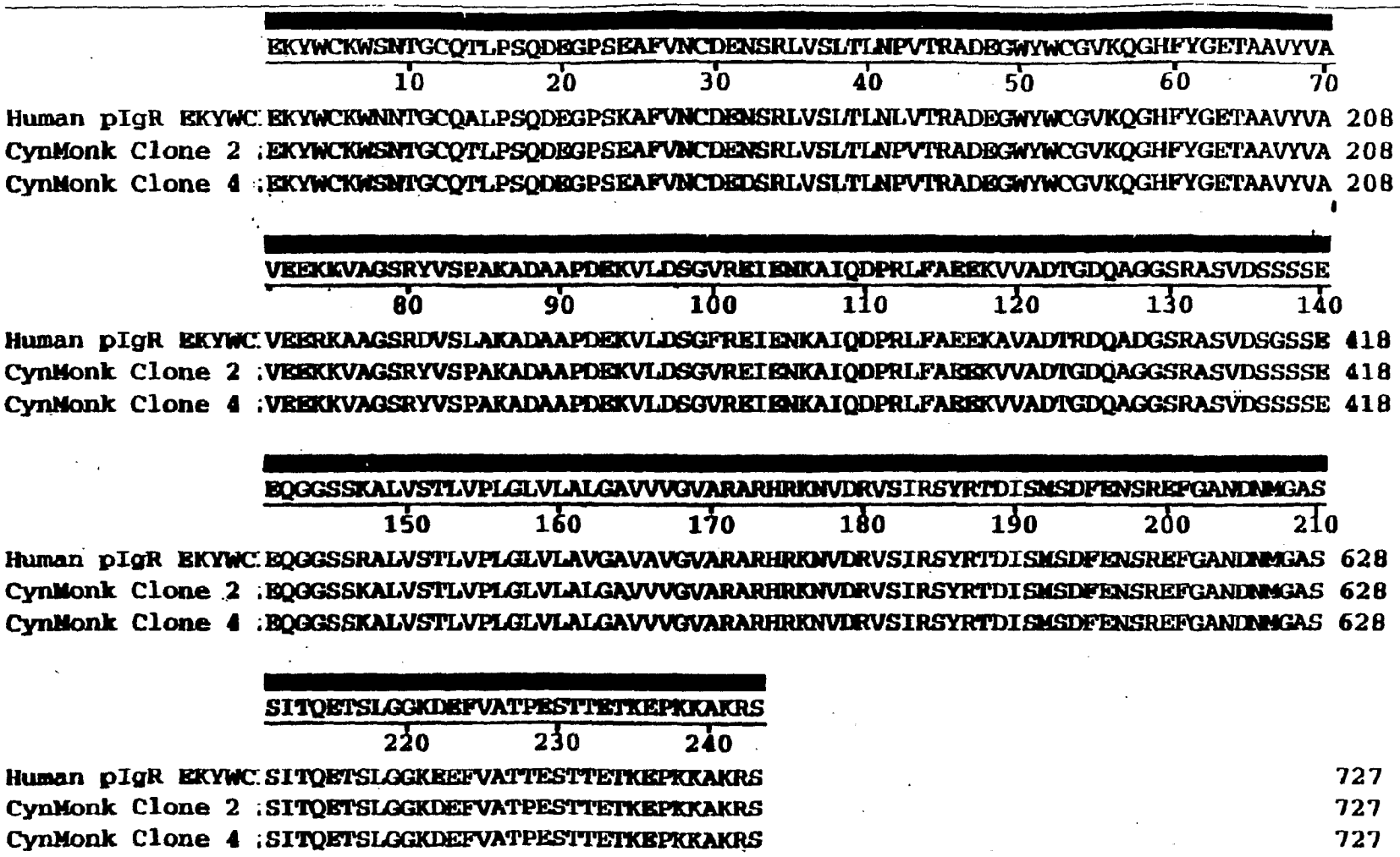

EKYWCKWSNTGCQTLPSQDEGPSEAFVNCDENSRLVSLTLNPVTRADEGWYWCGVKQGHFYGETAAVYVA
10 20 30 40 50 60 70
Human pIgR EKYWC: EKYWCKWNNTGCQALPSQDEGPSKAFVNCDENSRLVSLTLNLVTRADEGWYWCGVKQGHFYGETAAVYVA 208
CynMonk Clone 2 : EKYWCKWSNTGCQTLPSQDEGPSEAFVNCDENSRLVSLTLNPVTRADEGWYWCGVKQGHFYGETAAVYVA 208
CynMonk Clone 4 : EKYWCKWSNTGCQTLPSQDEGPSEAFVNCDEDSRLVSLTLNPVTRADEGWYWCGVKQGHFYGETAAVYVA 208
VEEKKVAGSRYVSPAKADAAPDEKVLDSGVREIENKAIQDPRLFAEEKVVADTGDQAGGSRASVDSSSSE
80 90 100 110 120 130 140
Human pIgR EKYWC: VEERKAAGSRDVSLAKADAAPDEKVLDSGFREIENKAIQDPRLFAEEKAVADTRDQADGSRASVDSGSSE 418
CynMonk Clone 2 : VEEKKVAGSRYVSPAKADAAPDEKVLDSGVREIENKAIQDPRLFAEEKVVADTGDQAGGSRASVDSSSSE 418
CynMonk Clone 4 : VEEKKVAGSRYVSPAKADAAPDEKVLDSGVREIENKAIQDPRLFAEEKVVADTGDQAGGSRASVDSSSSE 418
EQGGSSKALVSTLVPLGLVLALGAVVVGVARARHRKNVDRVSIRSYRTDISMSDFENSREFGANDNMGAS
150 160 170 180 190 200 210
Human pIgR EKYWC: EQGGSSRALVSTLVPLGLVLAVGAVAVGVARARHRKNVDRVSIRSYRTDISMSDFENSREFGANDNMGAS 628
CynMonk Clone 2 : EQGGSSKALVSTLVPLGLVLALGAVVVGVARARHRKNVDRVSIRSYRTDISMSDFENSREFGANDNMGAS 628
CynMonk Clone 4 : EQGGSSKALVSTLVPLGLVLALGAVVVGVARARHRKNVDRVSIRSYRTDISMSDFENSREFGANDNMGAS 628
SITQETSLGGKDEFVATPESTTETKEPKKAKRS
220 230 240
Human pIgR EKYWC: SITQETSLGGKEEFVATTESTTETKEPKKAKRS 727
CynMonk Clone 2 : SITQETSLGGKDEFVATPESTTETKEPKKAKRS 727
CynMonk Clone 4 : SITQETSLGGKDEFVATPESTTETKEPKKAKRS 727

FIGURE 2D

Pelb/5AF/myc/6HIS

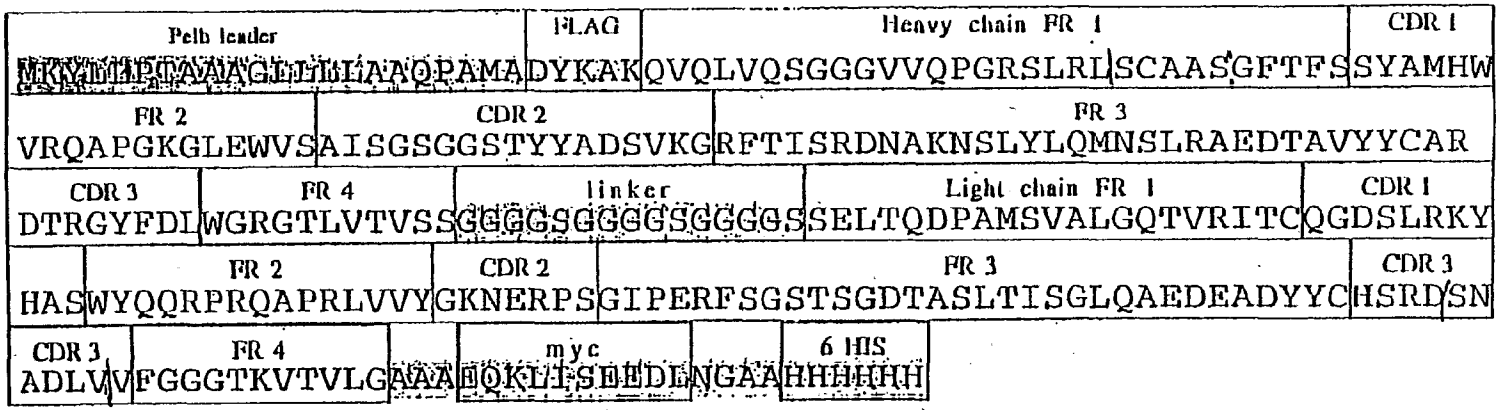



**FIGURE 3**

**Partial sequence, CbpA adhesin protein from *Streptococcus pneumoniae***

SWISS-PROT Accession Number O30874; SEQ ID NO:17; Length, 663 amino acids; Molecular Weight, 75064 Da

```
        10         20         30         40         50         60         70         80         90
         |          |          |          |          |          |          |          |          |
ENEGSTQAAT SSNMAKTEHR KAAKQVVDEY IEKMLREIQL DRRKHTQNVA LNIKLSAIKT KYLRELNVLE EKSKDELPSE IKAKLDAAFE

       100        110        120        130        140        150        160        170        180
         |          |          |          |          |          |          |          |          |
KFKKDTLKPG EKVAEAKKKV EEAKKKAEDQ KEEDRRNYPT NTYKTLELEI AEFDVKVKEA ELELVKEEAK ESRNEGTIKQ AKEKVESKKA

       190        200        210        220        230        240        250        260        270
         |          |          |          |          |          |          |          |          |
EATRLENIKT DRKKAEEEAK RKADAKLKEA NVATSDQGKP KGRAKRGVPG ELATPDKKEN DAKSSDSSVG EETLPSSSLK SGKKVAEAEK

       280        290        300        310        320        330        340        350        360
         |          |          |          |          |          |          |          |          |
KVEEAEKKAK DQKEEDRRNY PTNTYKTLDL EIAESDVKVK EAELELVKEE AKEPRDEEKI KQAKAKVESK KAEATRLENI KTDRKKAEEE

       370        380        390        400        410        420        430        440        450
         |          |          |          |          |          |          |          |          |
AKRKAAEEDK VKEKPAEQPQ PAPATQPEKP APKPEKPAEQ PKAEKTDDQQ AEEDYARRSE EEYNRLTQQQ PPKTEKPAQP STPKTGWKQE

       460        470        480        490        500        510        520        530        540
         |          |          |          |          |          |          |          |          |
NGMWYFYNTD GSMATGWLQN NGSWYYLNAN GAMATGWLQN NGSWYYLNAN GSMATGWLQN NGSWYYLNAN GAMATGWLQY NGSWYYLNSN

       550        560        570        580        590        600        610        620        630
         |          |          |          |          |          |          |          |          |
GAMATGWLQY NGSWYYLNAN GDMATGWLQN NGSWYYLNAN GDMATGWLQY NGSWYYLNAN GDMATGWVKD GDTWYYLEAS GAMKASQWFK

       640        650        660
         |          |          |
VSDKWYYVNG SGALAVNTTV DGYGVNANGE WVN
```

**FIGURE 4**

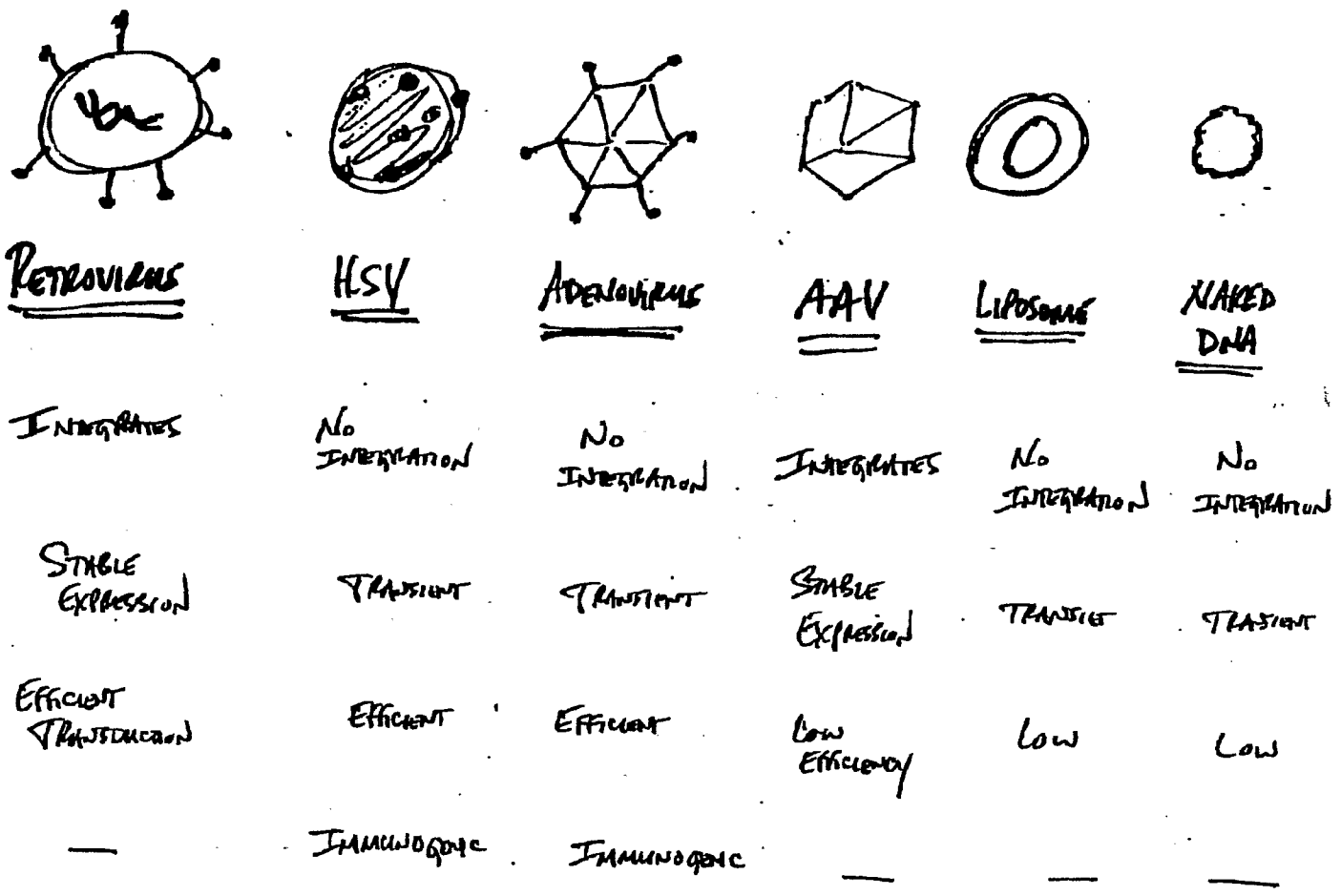



FIGURE 5 General characteristics of gene delivery systems.

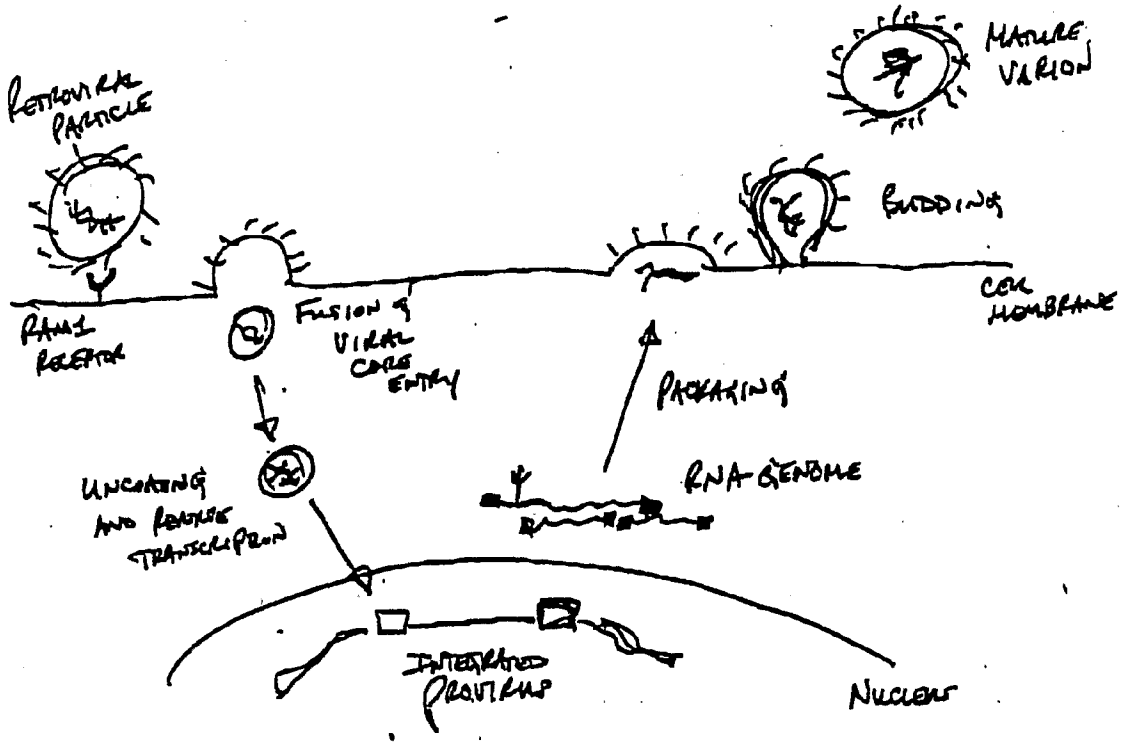



FIGURE 6 Schematic of the retroviral life cycle.

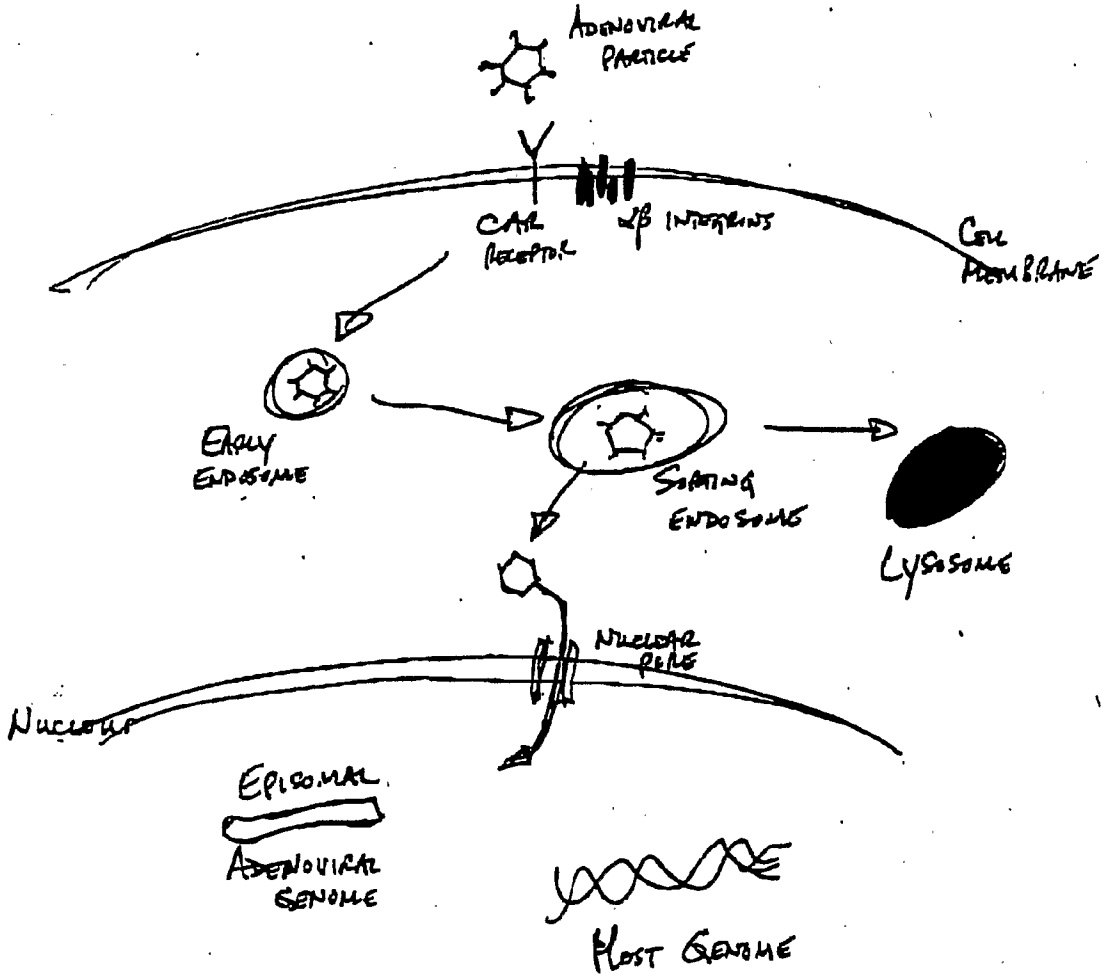



FIGURE 7 Schematic of adenoviral internalization and trafficking to the nucleus.

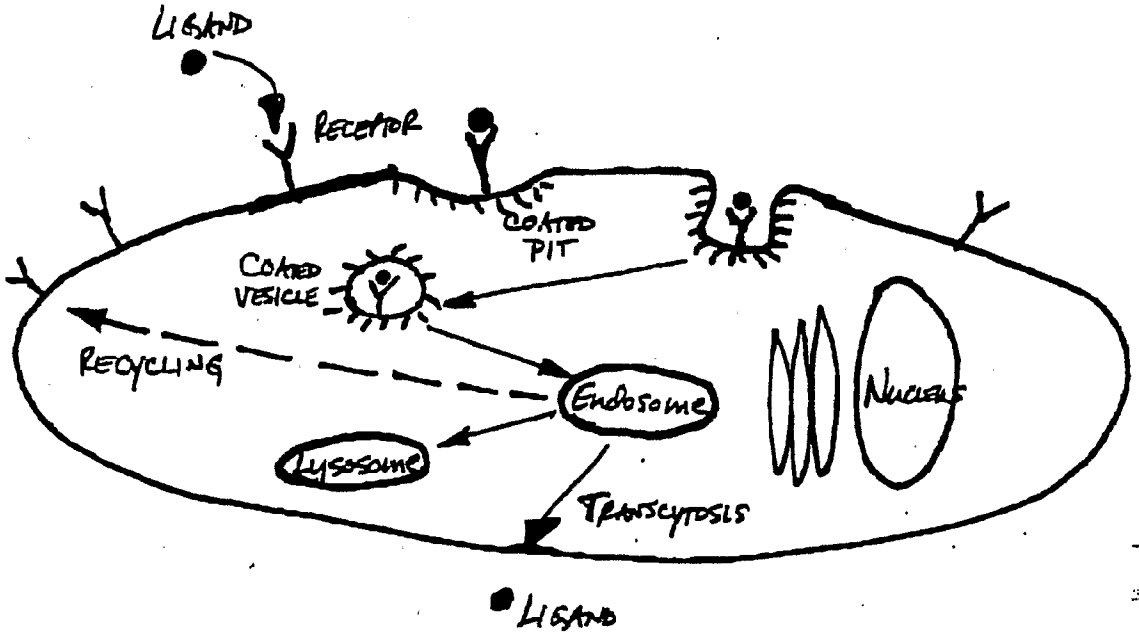



FIGURE 8 General schematic of receptor mediated endocytosis.

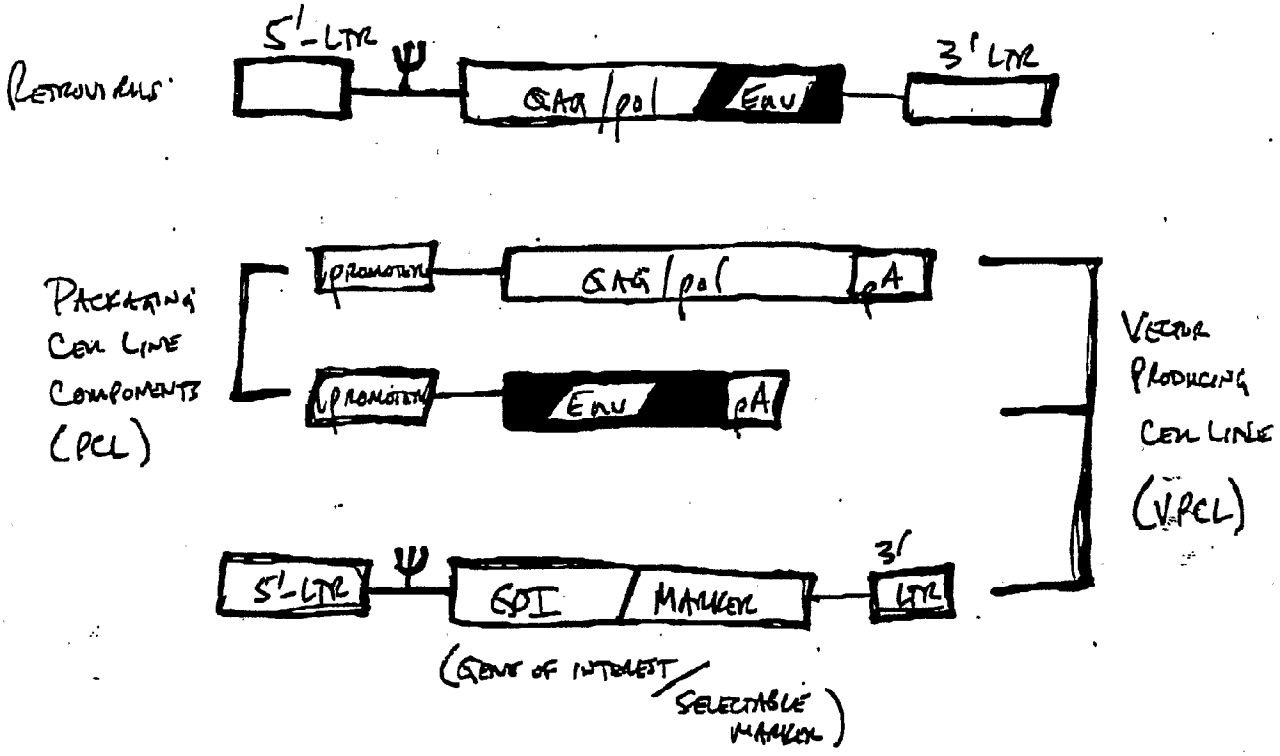



FIGURE 9 Packaging and vector producing cell line components.

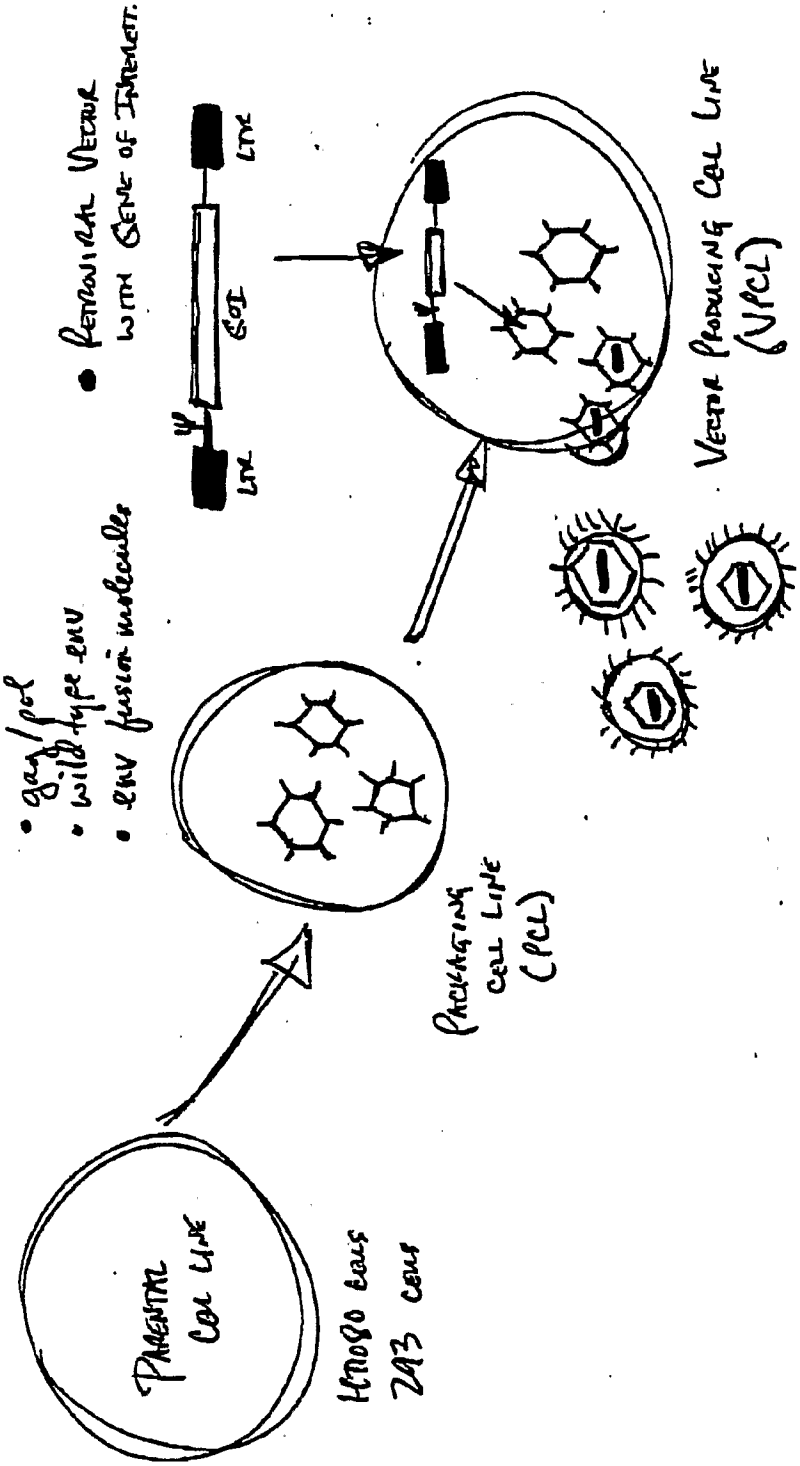



FIGURE 10 General strategy for the production of retroviral packaging and vector producing cell line components.

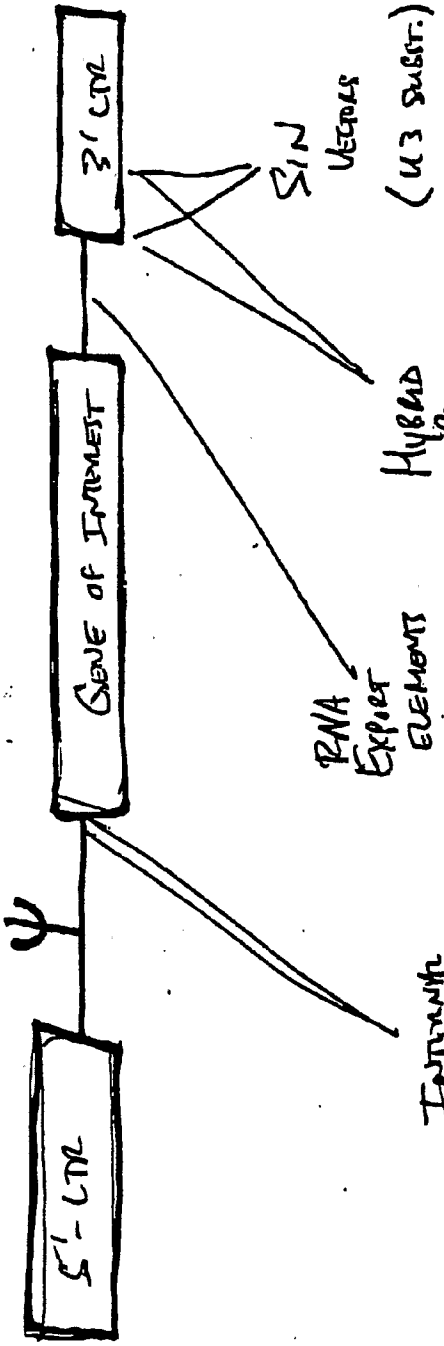



FIGURE 11 Basic retroviral vectors and various engineering derivatives.

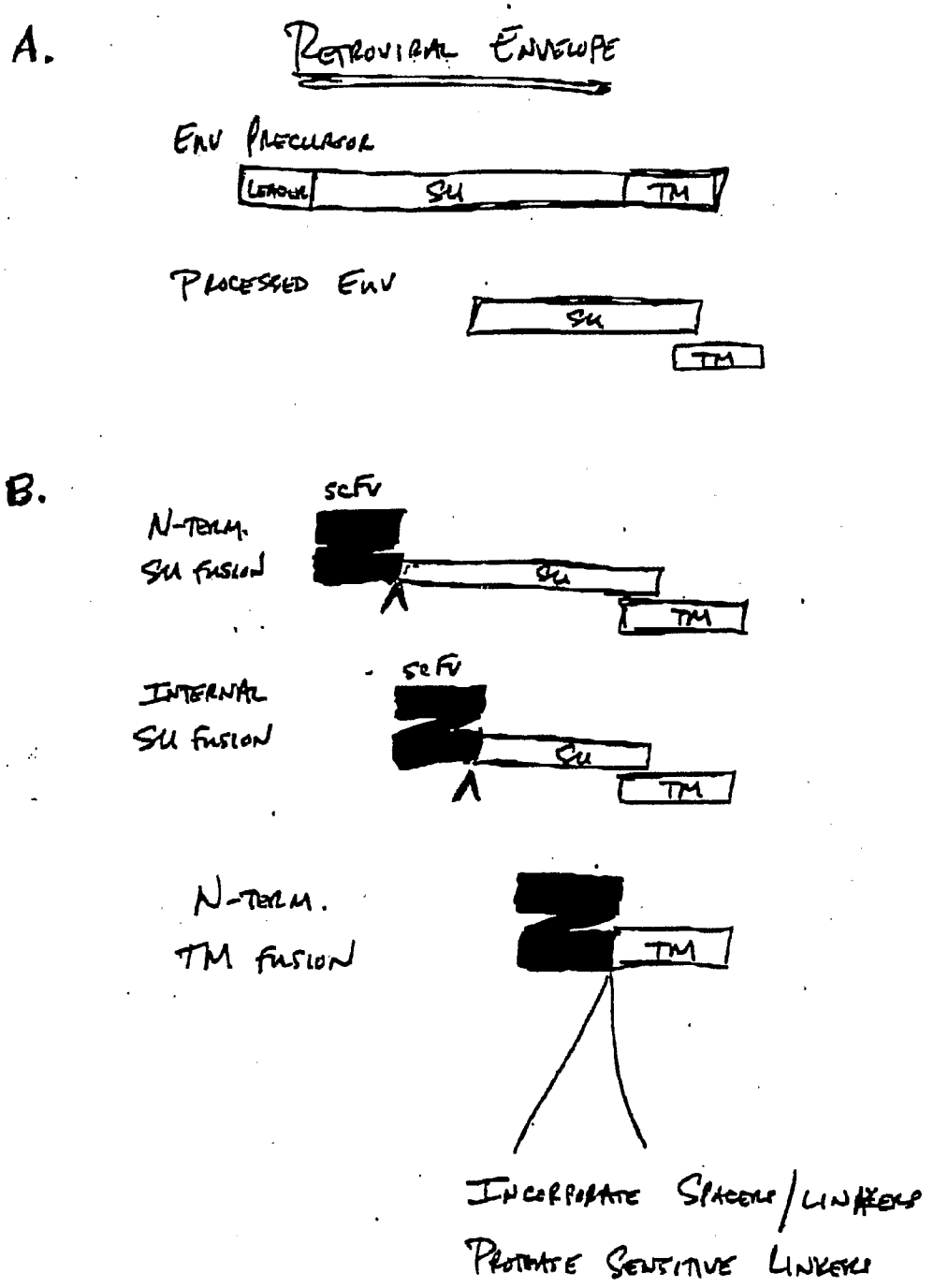



FIGURE 12 Retroviral envelope genetic fusion strategies.

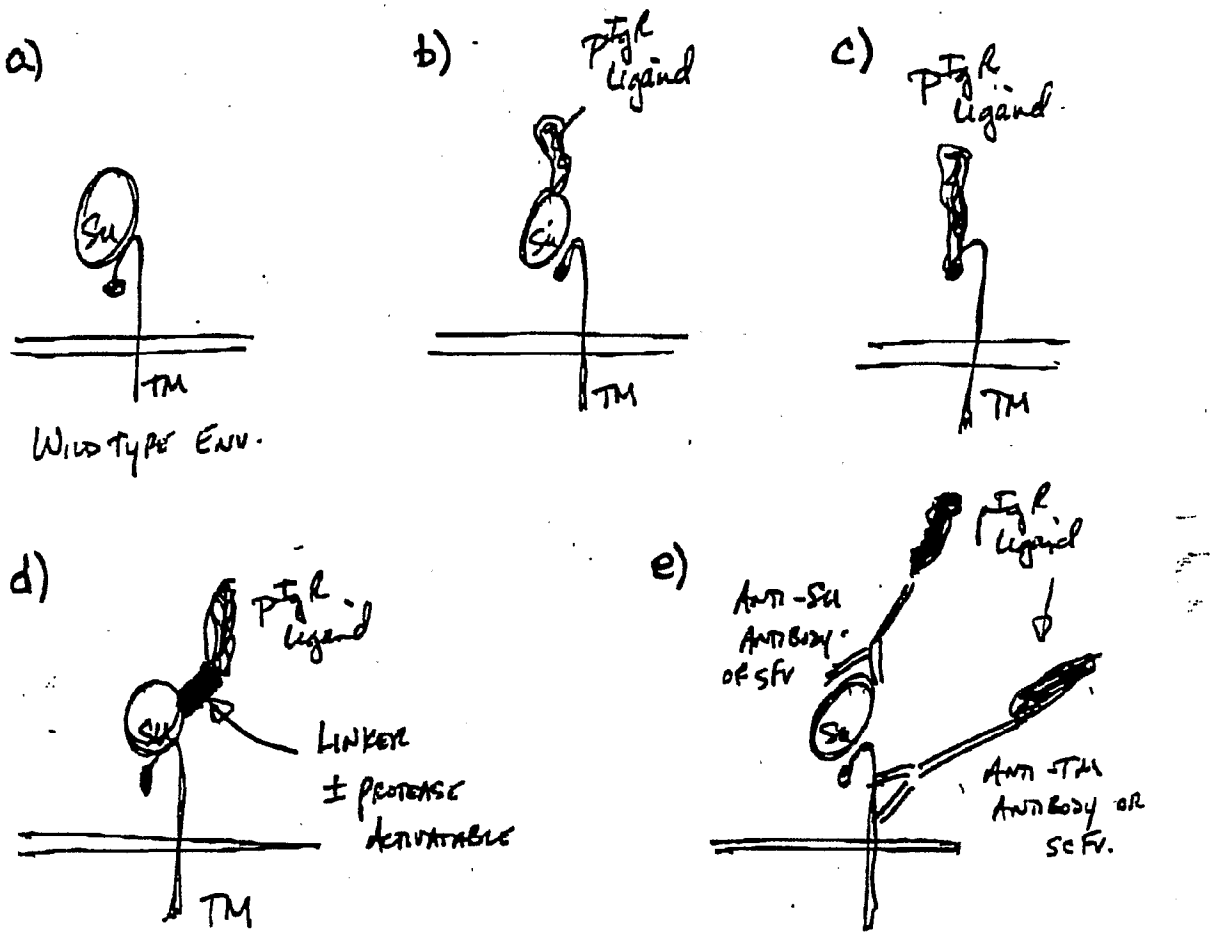



FIGURE 13 Re-targeting strategies using chimeric retroviral envelope fusion molecules or bi-specific targeting ligands.

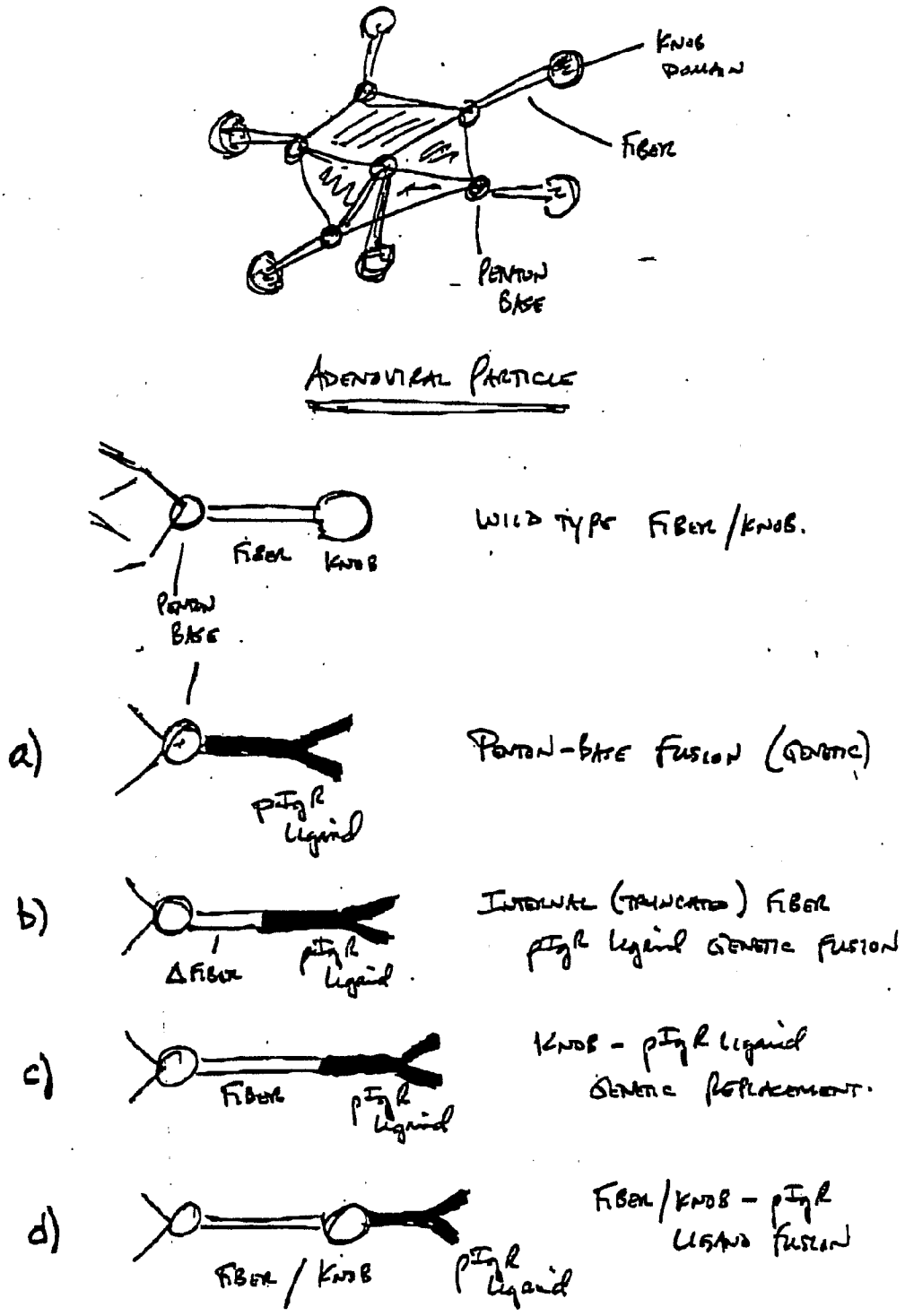



FIGURE 14 Adenoviral genetic fusion strategies.

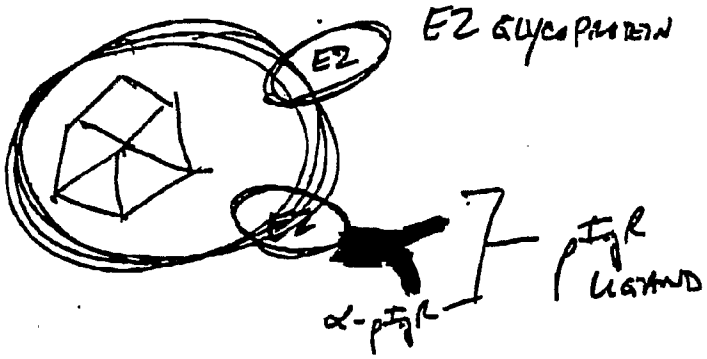



FIGURE 15 Targeting of alphavirus-based gene therapy vectors: re-targeting of recombinant Sindbis virus through the use of an E2 glycoportein fusion protein.

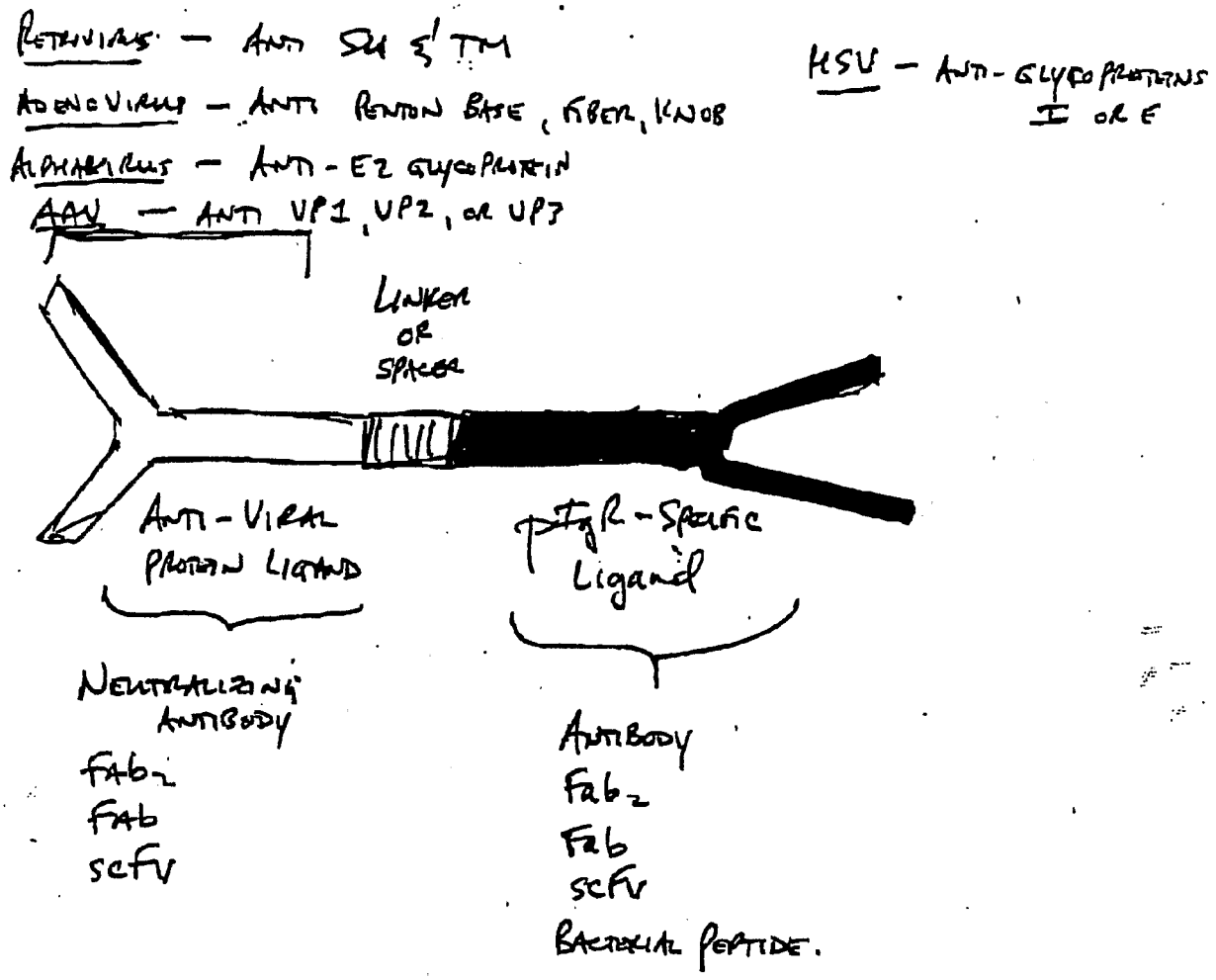



FIGURE 16 Schematic depicting pIgR-specific bi-specific antibodies or diabodies with specificity for selected viral protein epitopes for re-targeting of viral vectors.

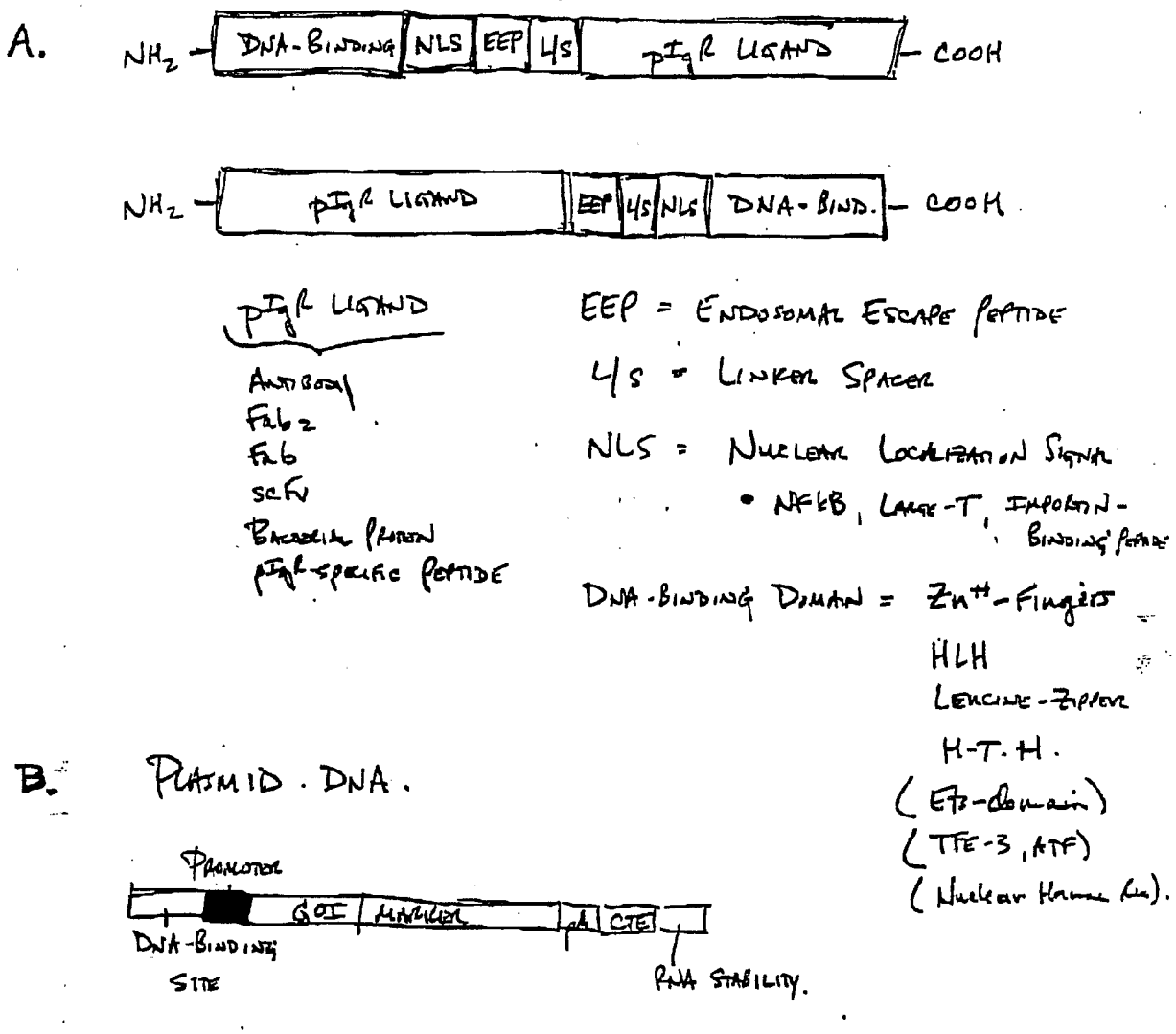



FIGURE 17 Naked DNA vectors and targeted delivery strategies. A. Schematic representation of genetically engineered pIgR binding proteins aimed at naked (plasmid) DNA delivery, in which the pIgR ligand would be fused in-frame to various protein domains for the facilitation of specific cellular functions such as DNA-binding (DNA-binding), endosomal escape (EEP, endosomal escape peptide), and nuclear transfer or residence (NLS, nuclear localization signal). These protein domains can be located in various orientations relative to the pIgR ligand, and different examples for each are shown. B. A linear schematic of a plasmid which would be delivered by the recombinant genetic fusion targeting molecule described in A. This plasmid would contain a specific transcription factor DNA-binding site, general or tissue-specific enhancer and promoter *cis* transcription elements, an efficient polyadenylation signal (pA), an RNA cytoplasmic transport element (CTE), and additional RNA stability elements.